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Genetic Manipulation of Allelopathy in Crop Species

Heather Ray ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Department of Genetics

Edmonton, Alberta

Fall 1993

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Heather Ray

10535-76 Ave.,
Edmonton, Alberta,
T6E 1L2
Canada

Aug. 25/93

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Reseach for acceptance, a thesis entitled "Genetic Manipulation of Allelopathy in Crop Species" submitted by Heather Ray in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr. P.J. Hastings
P.J. Hastings

Dr. W. Addison
W R Addison

Dr. E.A. Cossins
E A Cossins

Dr. R. Hammerschmidt
R Hammerschmidt

Dr. J. Kuspira
J. Kuspira

Dr. A.A. Szalay
A.A. Szalay

aug 19/93

Thesis Abstract

Plants may be affected by chemicals released by other plants, in the phenomenon of allelopathy. Frequently the effect is deleterious. Allelopathy may contribute substantially to crop yield losses caused by weeds. Common allelopathic compounds are phenolic acids and coumarins.

Two genetic approaches to reducing this injury are to increase the allelopathic potential of the crop plants and to increase the tolerance of the crop plants to allelochemicals produced by weeds.

Injury caused by various phenolic acids, coumarins, and weed extracts in flax, wheat, barley and oats was examined. Two approaches to increasing crop tolerance to some allelopathic compounds were examined. In the first, varieties of flax and barley were compared in the presence of phenolic compounds or wild oat extract. Significant intraspecific variation was shown. This variation is presumally genetic and hence subject to selection by conventional crop breeding.

In the second approach, genes likely to confer ability to detoxify allelochemicals were sought in several non-plant sources. A survey of potential sources was carried out. Two cytochrome P450 genes from mouse were chosen for plant transformation. P450IIA5 hydroxylates coumarin to umbelliferone, a compound much less toxic to plants. P450IA1 hydroxylates a variety of compounds, and seemed likely to hydroxylate phenolic acids. These genes were transferred into the Ti plasmid of *Agrobacterium tumefaciens* under the control of the bidirectional *mas* promoter, together with luciferase (*lux*F), which emits photons, and NPTII, which confers kanamycin resistance. The plasmids were transformed into tobacco and flax, which were selected for kanamycin resistance and screened for *lux*F expression. Presence of the P450 genes was confirmed by Southern blotting. Transcription was confirmed by Northern blotting. Functional assays of P450 enzyme activity have been uniformly negative, although activity of a native plant P450 has been detected. No phenotypic changes in the presence or absence of phenolic compounds were found. Possible reasons for this were explored.

Acknowledgements

This thesis has been pursued with the assistance of a good number of people. Financial assistance was provided by the Canadian Wheat Board through their scholarship program. Assistance in the laboratory has been provided very frequently by T. Hsu and by several summer students, and particular items of equipment by Dr. J. Weiner (Biochemistry) and Dr. A. Good. Dr. G. Bagy (Plant Science) examined phosphorylation of microsomal fractions of plants. Statistical advice was provided by A. Kilvert. Dr. A. McHughen (University of Saskatchewan) and Dr. J. Draper (University of Leicester) provided advice concerning regeneration of transformed flax. Dr. J. O'Donovan (Alberta Environmental Centre, Vegreville) provided advice including unpublished data on Alberta weeds. Dr. D. Pompon (Université Pierre et Marie Curie) and Dr. M. Negishi (National Institute of Health, North Carolina) donated the P450 genes used for plant transformation and antibodies against them. Dr. J. Kuspira closely edited the manuscript. Dr. W. Addison gave practical assistance with Northern blotting. Dr. A. Szalay (Plant Science) donated the key *Agrobacterium* and luciferase genes used, together with the use of luminometry equipment, and various members of his laboratory, particularly Dr. W. Langridge and A. Szenthe, provided frequent assistance. Dr. P. Hastings provided the initial impetus for the project and has provided continual and various support.

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List of Abbreviations

4CL = 4-coumaryl ligase
2,4-D = 2,4-dichlorophenoxyacetic acid, an artificial analogue of IAA
ABA = abscisic acid, a plant hormone
ANOVA = analysis of variance
ATP = adenosine triphosphate
BAP = benzylaminopurine, a plant hormone
BCIP = 5-bromo-4-chloro-3-indolyl-phosphate
cAMP = cyclic adenosine mononucleotide
CHS = chalcone synthase
CIP = calf intestinal phosphatase
CoA = coenzyme A
CHO = cinnamate 4-hydroxylase
CIP = calf intestinal phosphatase
coh = coumarin hydroxylase, P450IIA5 enzyme
cv = cultivar, cultivated variety
DMSO = dimethylsulfoxide
EDTA = tetrasodium salt of diaminoethanetetraacetic acid
expt. = experiment
fer = ferulic acid
FMN =flavin mononucleotide
GA = gibberellic acid, a plant hormone
GUS = β-glucuronidase
ht. = height
IAA = indoleacetic acid, a plant hormone
LB = Luria-Bertani medium for culturing bacteria
LU = light units, $1.6x10^6$ photons/sec
MOPS = 3-(N-morpholino)propanesulfonic acid
MS = Murashige-Skoog medium for cultured plant material
n.a. = not applicable
NAA = naphthaleneacetic acid, artificial analogue of a plant hormone
NADPH = nicotinamide adenine dinucleotide diphosphate, reduced
NBT = Nitro blue tetrazolium
n.d. = not done
NEN = New England Nuclear
P1 = P450IA1 enzyme
P1 = promoter 1 of the bidirectional *mas* promoter
P2 = promoter 2 of the bidirectional *mas* promoter
PAL = phenylammonia lyase
pca = *p*-coumaric acid
PCE = phenol/chloroform/isoamyl alcohol extraction of DNA followed by salt/ethanol precipitation
s.e. = standard error
TAE = Tris-acetate buffer for agarose gel
TBS = Tris-buffered saline solution
TBST = TBS with Tween 20 detergent
Ti = tumour inducing plasmid of *Agrobacterium tumefaciens*
tr. = treated
un. = untreated
UV = ultraviolet
van = vanillic acid
YEPD = yeast extract/peptone/dextrose medium for culturing yeast

I. Introduction

In this thesis, possible genetic approaches to the question of allelopathy in agriculture are examined. Allelopathy refers to chemical interactions between plants. Allelopathic compounds, from weeds or from crop residues, may severely reduce yield of crop species.

Developing crop varieties with greater toxicity to weeds, or with greater resistance to the effects of toxins from weeds, are potential approaches to reducing this yield loss. The first of these approaches has been very little investigated; the second not at all. This project examined various approaches to making crop species more resistant to allelochemicals produced by weeds.

Since no research had directly addressed this question, it was first necessary to generate background information on the response to allelochemicals of common western Canadian crop species. If genetic variation in response to allelochemicals could be shown to exist, selective breeding for increased resistance could be practised.

An alternative approach was to find foreign genes capable of detoxifying allelochemicals. Such genes could then be introduced by transformation into crop species. A large variety of potential gene donors existed. It was necessary to survey them to determine which species had good resistance to allelochemicals, and which genes conferring resistance were available. Once potential genes were identified, they could be integrated into the genomes of crop species. If these genes were correctly expressed in the plant background, they might reduce yield loss to allelochemicals and therefore to weeds.

Crop varieties with resistance to allelochemicals, however arrived at, should be useful to farmers in reducing crop yield losses to weeds. This might have various beneficial effects on farming practices.

In the first part of this thesis, allelopathic effects on flax (*Linum usitatissimum*), bread wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and oats (*Avena sativa*), using principally chemicals known to be produced by wild oats (*Avena fatua*), were examined. Intraspecific variation in resistance to allelochemicals in flax and barley was then determined. In the second part of the thesis, a variety of potential genetic determinants of resistance to allelochemicals were surveyed. Two P450 genes from mouse (*Mus musculus*), were chosen from this group. They were transformed into flax and tobacco (*Nicotiana tabacum*) and their expression, enzyme activities, and phenotypic effects determined.

1.1 Definition of allelopathy

Living and dead plants release chemicals which may influence the growth of other plants around them. This phenomenon of chemical interactions has been called allelopathy. Allelopathic interactions may be beneficial or deleterious. Sometimes the term has been confined to deleterious effects (Rice, 1974) but since the same chemicals at different doses or applied to different plants may cause benefit or injury, this distinction is inconvenient to maintain. However, injury appears to be the more common effect and is the more studied.

Molisch, who coined the term allelopathy, included in it effects caused by and on microorganisms, and benefit and injury among plants (Rice, 1984). Rice in 1974 limited the use of the term to deleterious effects but later agreed with Molisch (Rice, 1984). Allelopathic compounds produced by plants may also deter grazing by insects and other herbivores, and the term allelopathy is now appearing in these contexts also (Waller, 1989). In this thesis, however, allelopathy will refer only to chemical interactions between plants unless noted otherwise.

Allelopathy is distinct from competition for resources (light, water, minerals,

"growing space") but their effects are difficult to distinguish experimentally. This has led to widespread confusion and sometimes to doubt of the existence of allelopathy in nature. The assumption, formerly frequent among plant scientists, that all injury done by one plant to another is due to competition, is often false. Greater clarity results from the use of the terms:
interference to mark the sum total of effects of plants on each other;
competition to mark those effects which result from withdrawal of some factor normally present (e.g. light);
allelopathy to mark those effects which result from addition of some factor, such as a toxin.

It should be noted that allelopathy and competition may interact. For example, a seedling injured by allelopathic compounds may be less able to compete effectively with other plants later.

1.2 History

Observant farmers have no doubt noticed the effects of allelopathy from prehistoric times. Many "primitive" farming systems incorporate some plants at least in part to control weeds (Liebman 1987). Written references to allelopathy may well begin with Theophrastus (c. 330 B.C.), and proceed through books of natural observations, herbals, and farming manuals. Such references are reviewed by Rice (1984) and by Willis (1985). Experimental study of allelopathy did not begin until the 1900s. The devising of methods to separate the effects of allelopathy from those of competition was and still remains highly important for such studies.

1.3 Importance of allelopathy in agriculture

The effects of weeds (unwanted plants) on crops (wanted plants) are the basis of a major part of agribusiness. Sales of tillage machinery and herbicides depend heavily on the existence and toughness of weeds.

Weed damage to crops may vary from 0 to 100%. The extent of damage varies with numerous factors: species of weed and crop, numbers, planting density, weather throughout the season, cultivation and harvest practices, seed cleaning, and much else. Very few fields escape some weed damage. A commonly cited figure for average loss of yield due to weeds is 10% where crops are well weeded (Chandler, 1985). Estimates of loss in several unweeded tropical crops run from 30 to 80% (Sen, 1988). Clearly, this represents an enormous amount of wasted plant material from the point of view of the farmer.

In some cases the presence of weeds may increase crop yield. Corn cockle, *Agrostemma githago*, appears to increase bread wheat (*Triticum aestivum*) yield when present in small numbers (quoted in Rice, 1984). Complex cropping systems may leave certain non-harvested plants to allow them to suppress other, more harmful weeds and for other reasons (Liebman 1987, Gliessman 1988).

Pestiferous weed in general are characterized by several traits.
They maintain a good bank of seeds or growing points in the soil at all times, such as random germination, reproductive stolons, non-uniform time of seed set and freely dehiscent seeds. Their hardiness and flexibility of response to prevailing stresses (drought, trampling, cold, etc.) is greater than that of the crop. They are usually more competitive than the crop, in terms of faster early growth, more profuse roots and greater shade cover (Holm et al., 1987). They are frequently toxic to the crop (Putnam and Weston, 1986) and may be resistant to toxins produced by the crop. Many of the world's worst weeds rate high on all these components of injury. Of the most prevalent

weeds of Alberta, as determined by the Alberta Weed Survey (J. O'Donovan, unpublished data, 1986-1988), six of eleven have been identified as allelopathic (Table 1). On those remaining no research has been done.

Hundreds of reports indicate that the presence of weeds reduces crop yield. In nearly every report this effect is attributed to competition. Few researchers, however, try to separate possible allelopathic effects from those of competition. Indeed this is difficult to do, particularly in the field where it is most relevant, since the same weed simultaneously withdraws resources and adds allelopathic compounds to the soil.

Whittaker (1970) suggested that: "the source of the chemical in the plant should be found, and the route it follows from release from one plant to effect on another traced. . . Identification of the chemical is to be sought, and the manner of inhibition of germination or growth determined. . . One finally wants to show. . . that the quantitative relations of the chemical agents identified as they occur in the soil are adequate to produce the observed degree of inhibition of other plants. . . " (quoted in Fuerst and Putnam, 1983). This roughly parallels Koch's postulates and is expounded in greater detail by Fuerst and Putnam (1983). They note that "allelopathic interference can be selectively eliminated by. . . using biotypes of the agent which lack the proposed toxin". In some crops, varieties which release more of particular compounds have been identified (Fay and Duke, 1977, Alsaadawi et al., 1986). A second genetic method is to use biotypes of the recipient species which are insensitive to the toxin.

By the criteria of Fuerst and Putnam, very few cases of proven allelopathy or competition exist, only numerous cases of interference. These criteria still omit some other interactions between factors which might affect interference; some of these are discussed by Qasem and Hill (1989).

A variety of methods have been adopted to study allelopathy or to distinguish its contribution to interference. In the replacement series, weed and crop species are interplanted in field plots in known proportions from 0:100 through 50:50 to 100:0, the total being constant. The yields of each component and the total yield are found. This allows an index of interference to be constructed (Dekker et al., 1983). This method permits inferences about allelopathy and competition. It has the advantage of being very close to normal field conditions. Selective herbicides may be used to kill weeds in the field, removing the competition but retaining the allelopathy. This method is simple, although it relies on allelopathic components of dead plants being the same as those from live plants. Dry or wet extracts of weeds may be added to crops. This can be done in field or laboratory. This method is subject to many interfering factors, such as microbial action on added material and changes in physical properties of the soil. These have sometimes been controlled by using sterile conditions or poplar mulch. It is simple and may be closely related to field conditions. Resins or activated charcoal may be added to soil or growth media to remove toxins. Use of resins in hydroponic culture can give estimates of release of allelochemicals from intact plants (Tang, 1986). In step-stair methods, root or shoot washes of one plant are used to water another plant. This has the advantage of using intact plants, but it is difficult to ensure that donor and recipient get exactly the same nutrients. These methods may be combined to decide whether a particular weed is allelopathic to a crop species.

Notably absent from previous research on allelopathy is any genetic approach. Only a few lines suggesting such an approach have been published.

Many weeds and weed residues have been shown to release compounds toxic to crop species. The commonest compounds isolated from weeds, phenolic acids and coumarins, have been shown to be toxic to many crop species. Phenolic acids and coumarins are taken up freely by plants and have a variety of toxic effects (Rice, 1984; Mandava, 1985; Putnam and Weston, 1986; Waller, 1989).

There have been few reports on actual levels of phenolic compounds in soil. The average molarity of phenolic acids in soil solution in four types of soil was 8.4×10^{-5}

Table 1. Worst weeds of Alberta by relative abundance (Alberta Weed Survey, unpublished data of J. O'Donovan, 1988) and their allelopathic potential. Allelopathic, +; possibly allelopathic, ?.

Rank	Weed			Reference
1	Stinkweed	*Thlaspi arvense*	+	this thesis
2	Wild buckwheat	*Polygonum convolvulus*		
3	Wild oats	*Avena fatua*	+	Schumacher et al (1983)
4	Chickweed	*Stellaria media*	?	Mann and Barnes (1950)
5	Lambsquarters	*Chenopodium album*	+	Bhowmik and Doll (1982)
6	Green foxtail	*Setaria viridis*	+	Bhowmik and Doll (1982)
7	Hemp nettle	*Galeopsis tetrahit*		
8	Field horsetail	*Equisetum arvense*		
9	Shepherd's purse	*Capsella bursa-pastoris*		
10	Russian thistle	*Salsola kali*	+	Lodhi (1979)
11	Canada thistle	*Cirsium arvense*	+	Stachon and Zimdahl (1980)

M (Whitehead, 1964), below the threshold of severe damage. Cultivated soil had the lowest phenolic concentration. Wang et al. (1967) also found phenolic acid levels in several soils just below the threshold of damage. However, when crop residues are incorporated as in no-till farming, soil phenolic levels may be much higher. Guenzi and McCalla (1967) reported up to 100 kg/ha total phenolic acid from several temperate crops, while Lodhi et al. (1987) reported 1500 kg/ha ferulic and *p*-coumaric acids from wheat under tropical conditions. Such levels will substantially damage crops. Levels available to following crops depend on many factors including soil type, weather, and micro-organisms. Bulk soil levels may be much lower than those found adjacent to residues or roots of live plants and weeds.

1.4 Chemicals involved in allelopathy

The chemicals implicated in allelopathy are nearly all secondary metabolites. Some of these are important in a variety of ways and in most plant species. For example, *p*-coumaric acid, which is allelopathic to many plants, is an intermediate in the biosynthesis of lignin, a variety of pigments and scent agents, and antimicrobial compounds known as phytoalexins.

Allelopathic chemicals belong to many chemical classes. The majority, however, and all those studied here, are derived from phenylalanine (occasionally tyrosine) by the phenylpropanoid pathway. The central pathway is shown in Figure 1. While this figure is far from inclusive, the compounds shown are among the most common allelopathic agents. Numerous others may be derived from them.

The hydroxybenzoic and cinnamic acid derivatives are composed of a benzene ring with a one- or three-carbon acidic side chain respectively. Generally, the most hydrophobic of these groups have the strongest effects on plants (Glass, 1974).

The coumarins are derived from cinnamic acid derivatives. If the *trans* isomer changes to *cis*, as occurs spontaneously or in the presence of light, any hydroxy group at position 9 on the ring will promptly react with the carboxy group to form a coumarin (Brown, 1985). Coumarins are generally more toxic than the acids, at least in part because they are more hydrophobic.

Flavonoids, isoflavonoids and flavonones are derived from the coenzyme A conjugate of *p*-coumaric acid. More than one chemical pathway may lead to the same compound. Which flavonoids are produced varies according to plant species and variety, tissue, state of growth, and other factors. Some of these compounds are toxic to plants. A great variety of other functions such as flavouring, scent, and pigment agents and as phytoalexins and grazing deterrents have been ascribed to different flavonoids and related compounds. Thousands of members of these classes have been isolated and described. Many are found in only a single genus.

The effects of allelochemicals on plants may include some or all of the following:

1. delay or prevention of seed germination;
2. inhibition of root and/or shoot elongation;
3. inhibition of chloroplast function and chlorophyll development;
4. inhibition of protein synthesis;
5. inhibition of cell division;
6. interference with, or mimicry of, effects of phytohormones;
7. mutagenicity; and
8. depolarization of membranes (Einhellig, 1986).

Thus a great variety of effects are produced. While it is difficult to distinguish primary and secondary effects, the depolarization of membranes by many allelopathic compounds is prompt and severe and could lead to many of the other effects. Interference with phytohormone function could disrupt normal plant growth. These are tentatively classed as the primary effects of allelopathic compounds on plants.

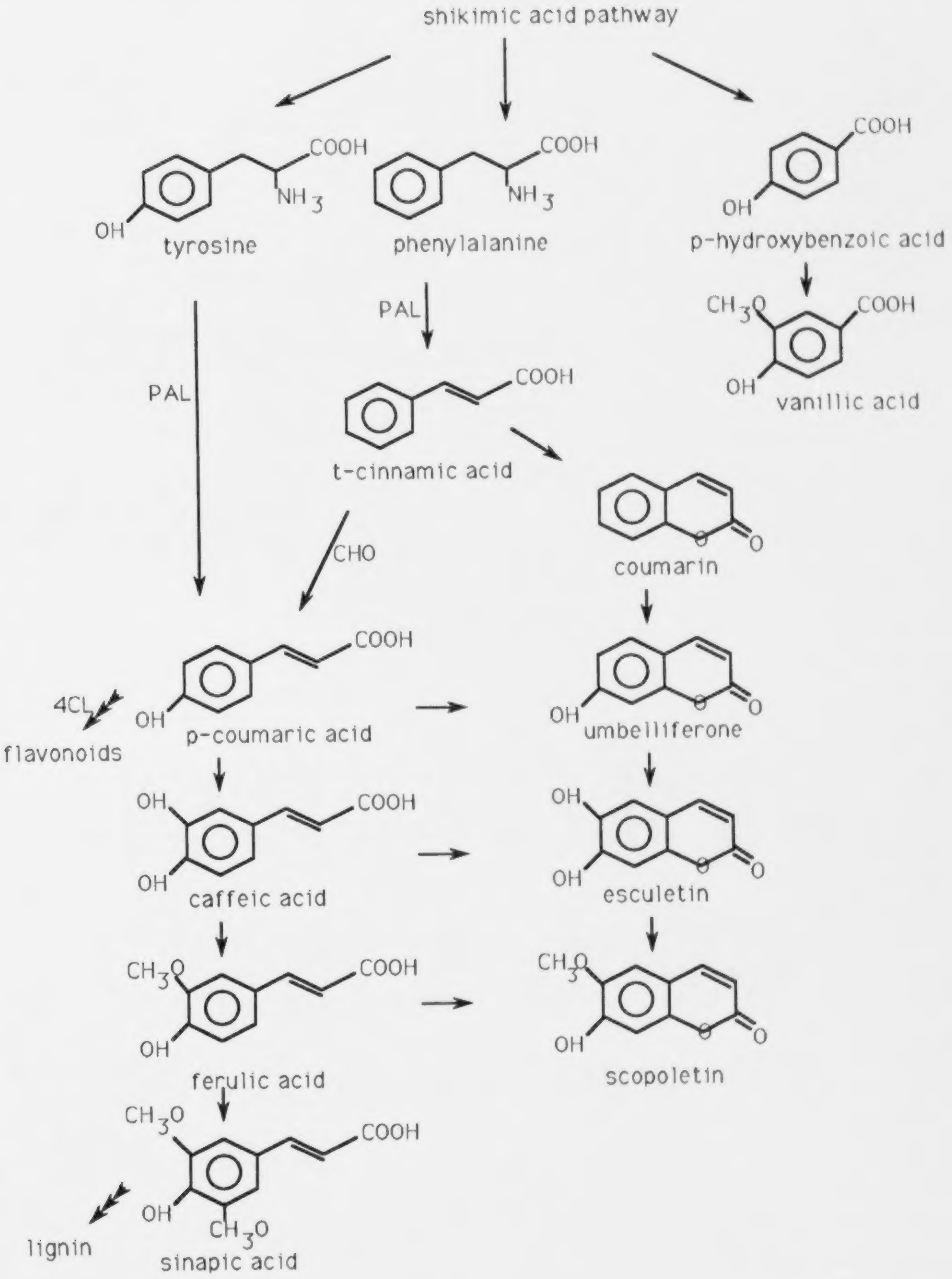

shikimic acid pathway
COOH
NH3
OH
tyrosine
COOH
NH3
phenylalanine
COOH
OH
p-hydroxybenzoic acid
PAL
PAL
COOH
t-cinnamic acid
CH3O
COOH
OH
vanillic acid
O
O
coumarin
CHO
COOH
4CL
OH
flavonoids
p-coumaric acid
OH
O
O
umbelliferone
OH
COOH
OH
caffeic acid
OH
OH
O
O
esculetin
CH3O
COOH
OH
ferulic acid
CH3O
OH
O
O
scopoletin
CH3O
COOH
OH
CH3O
lignin
sinapic acid

Figure 1. The phenylpropanoid pathway. PAL is phenylalanine ammonia lyase; 4CL is p-coumaryl ligase; CHO is cinnamate hydroxylase.

Membranes depolarize promptly on addition of any of several benzoic and cinnamic acid derivatives to excised barley roots (Glass and Dunlop, 1974). Uptake of potassium and phosphate stops or reverses immediately (Glass, 1974; Harper and Balke, 1981). The 50% uptake inhibition level varies with hydrophilicity, as measured by octanol:water partition coefficient (Glass, 1974; 1975). Respiration and mitochondrial function are severely affected (Demos et al., 1975), probably as a consequence, as are numerous other membrane-dependent functions.

Phenolic compounds interact with plant hormones in numerous and sometimes contradictory ways (Einhellig, 1986). Many interact with indoleacetic acid oxidase (IAA oxidase), either inhibiting or promoting its activity, and thereby affecting auxin levels. Scopoletin and coumarin increase IAA levels; umbelliferone decreases them (Andreae, 1952; Neumann, 1959; Mandava, 1979). 2-, 3-, or 4- hydroxybenzoic acids reduce IAA levels, while 3,4-dihydroxybenzoic acids increase IAA levels (Lee and Skoog, 1965; Mandava, 1979). The pattern followed by benzoic and cinnamic acids, coumarins and probably flavonoids is, with exceptions, as follows: no substitutions on the phenolic ring leads to increased auxin levels by inhibition of IAA oxidase; *para*-substitution leads to decreased auxin; *para*- and *meta*-substitution leads to increased auxin; and *para*- and *ortho*-substitution leads to decreased auxin (Gortner and Kent, 1958; Lee and Skoog, 1965; Kefeli, 1978; Grambow and Langenbeck-Schwich, 1983; Mandava, 1979; Einhellig, 1986). Flavonoids are also reported to regulate auxin transport (Jacobs and Rubery, 1988).

Phenolic compounds also counter the actions of kinetin, gibberellic acid (GA) and abscisic acid (ABA) (Kefeli and Kutacek, 1977; Laloraya et al., 1986; Ray, 1986). Some of these interactions are light-dependent (Grambow and Langenbeck-Schwich, 1983). Coumarin and other compounds may stimulate ethylene production (Mandava, 1979), possibly as a consequence of auxin interactions. Ray (1986) suggested a role for phenolics in "balancing the effect of inhibitors/promoters during plant growth". That is, native phenolic compounds may be integrated into normal phytohormone networks or serve in signal transduction.

A variety of effects of phenolics on production of particular enzymes has been reported. *T*-cinnamic and *p*-coumaric acid, early compounds in phenylpropanoid production, affect the activity of enzymes in this pathway via an unknown factor binding upstream of, for example, the chalcone synthase promoter (Loake et al., 1991). Salicylic acid and related compounds also affect the wound response (Doherty et al., 1988), while various phenolic compounds affect the incorporation of phenylalanine into proteins (Van Sumere et al., 1972).

Coumarin and other phenolic compounds may strongly reduce or delay germination (Van Sumere et al., 1972); this effect may be incidental, or may be treated by the seed as an environmental signal indicating unsuitable growing conditions. Use of such a signal would delay germination in the immediate vicinity of growing or decaying plants.

Coumarin is also reported to arrest cells in mitosis, similarly to colchicine (Mandava, 1979). A large variety of phenolic compounds inhibit cell division. Some phenolic compounds are mutagenic. This is not characteristic of phenolic acids and coumarins, but of particular more complex phenolic compounds, such as the linear furanocoumarins.

To be effective, allelopathic compounds must be released by one plant and taken up by another. Several routes are possible. Volatile compounds may diffuse through the air. Chemicals may be washed off leaves by rain or diffused or actively exuded from roots. Litter from the plant, or the whole dead plant, eventually rots in the soil, releasing component chemicals which may be allelopathic. Soil particles may adsorb and inactivate, and later release, phenolic compounds. Soil water leaches them away. Soil microbiology changes in response to allelochemicals; some microbes are inhibited, while others detoxify or metabolize these compounds, and others produce more toxic ones of their own (Einhellig, 1986). All these factors may alter allelopathic effects.

Allelopathy may affect the source plants as well as other plants. Consequently, they are sometimes compartmentalized within the cell or the plant. Sweet clover (*Melilotus alba*) cells contain glycosylated *o*-hydroxycinnamic acid in the vacuole. When crushed or attacked by mould, the vacuolar membrane is broken and a cytoplasmic β-glucosidase removes the glycosyl group. In the presence of light and in acidic solution the free acid is converted to the highly toxic allelochemical coumarin (Berkenkamp, 1971).

Numerous factors affect levels of phenolic compounds found in plants. These factors may broadly be characterized as developmental and stress-related. Presumably internal levels have some relationship to levels which are released to the environment from the live plant, and all such compounds will eventually be released after its death. . Phenolic compounds are required for the biosynthesis of a large number of plant components, such as lignin, pigments, and scent agents. Their synthesis varies between tissues and developmental stages.

Many phenolic compounds implicated in allelopathy have also been identified as phytoalexins, low molecular weight compounds which increase rapidly on infection by various fungi, bacteria and viruses (Dixon, 1986). Phenolic compounds also increase following wound damage to the plant, caused mechanically or by large grazers or phytophagous insects (Kogan and Paxton, 1983; Reichardt et al., 1988). In some cases, levels of particular phenolic compounds correlate well with resistance to particular insect pests (Berkencamp, 1971; Hedin et al., 1983; Classen et al., 1990). In others, no correlation appears (Trumble et al., 1990), but as the plant may have large arrays both of defenses and enemies, this is not unexpected.

Ultraviolet light induces phytoalexin production in many plants (Koeppe et al., 1969). The case of parsley (*Petroselinum crispum*) has been well characterized (Lois et al., 1989). Flavonoids and other compounds produced in response to ultraviolet light absorb at short wavelengths and so protect plant cells from damage. Ordinary light also induces phenylpropanoid production but less strongly (Hrazdina and Creasy, 1979; Engelsma, 1979). Strong light on *Brassica* species makes their residues more toxic (Mason-Sedun and Jessop, 1989).

Responses to other stresses such as heat, drought, nutrient deficiencies and air pollution occur, as measured by increased toxicity of residues (Mason-Sedun and Jessop, 1989) or accumulation of various phenolic compounds (Gershenzon, 1984; Kogan and Paxton, 1983; Dercks et al., 1990). Some fungicides and herbicides increase phenolic production (Cartwright et al., 1980; Koeppe et al., 1969). In these cases the effect of the applied compound might be at least in part through activating the plant's own defense mechanisms.

Phenolic acids appear to regulate their own production in a concentration-dependent manner. Exogenously applied *t*-cinnamic acid at less than 10^{-4} M increased expression from the bean (*Phaseolus vulgaris*) chalcone synthase promoter, while higher concentrations inhibited it, and *p*-coumaric acid at the same (higher) levels increased expression (Loake et al., 1991). In tissue culture, high levels of auxin on flax resulted in decreases in *p*-coumaric and ferulic acids, and the appearance of *p*-hydroxybenzoic and vanillic acids not previously seen in this species, while high levels of kinetin had the opposite effect (Ibrahim and Shaw, 1970).

Because of the numerous inducing factors present under usual growth conditions in the field, levels of allelopathic compounds produced by field crops and weeds are probably well above minimal levels.

Genetic control of phenylpropanoid metabolism involves numerous mechanisms, as would be expected for the large number of roles which phenolic compounds fill. Some genes of general phenolic metabolism are among the best characterized of plant genes, particularly phenylalanine ammonia lyase (PAL), *p*-coumaryl CoA ligase (4CL) and chalcone synthase (CHS). These are important control points, PAL into general phenylpropanoid metabolism, 4Cl towards lignins and flavonoids, and CHS towards flavonoids and related compounds (away from lignins). Multiple copies of these key genes are common (Harker et al., 1990; Lois et al., 1989). Some of their promoters contain numerous upstream regulatory elements, often responding to several different stresses. Promoter analysis of parsley 4CL showed regions required for UV induction and developmental expression patterns; there are at least six footprints within 500 base pairs of the start of transcription (Hauffe et al., 1991). Other identical or near-identical copies of the same gene may be induced by different stresses or developmental signals, reducing the number of factors to which a single gene has to respond and increasing flexibility of control. Maize (*Zea mays*) has two CHS genes; *C2*, responsible for flavonoid production in a number of tissues, and *Whp*, responsible for flavonoid production in pollen in the absence of *C2* (Franken et al., 1991). In this case, the RNA levels produced by the two genes are very similar but protein levels differ greatly if a third gene, anthocyanin intensifier (*in*), is expressed. These workers suggest that, since the exons are nearly identical and can replace each other in the absence of *in*, the 5' non-translated region of the mRNAs, which differ in sequence and potential secondary structure, may act as markers for differential regulation by the product of *in*. Another unusual control element is found in parsley (Douglas et al., 1991), where exon sequences are necessary for induction by elicitor and UV, although not for tissue-specific expression. This is so far unique among genes transcribed by RNA polymerase II.

The importance of phenolic metabolism to plants suggests caution in altering it, as the possibility of deleterious secondary effects exists. This importance also makes clear the desirability of genetically examining more of its aspects.

1.5. Modifying allelopathy in crop/weed interactions

Allelopathy in the field has several components. Allelopathic chemicals produced by either crop or weed may be toxic to the crop itself, to subsequent crops, or to weeds. Since weeds are unwanted and unpredictable, modifying allelopathy in the field must take place by manipulating the crop or the way it is managed. Many cultivation practices are relevant to weed control, but none of these will be discussed.

Three chief modifications of the crop might be used in order to improve weed control:

1. plant characteristics such as early germination and establishment and rapid development of a complete canopy may be selected;
2. the crop plant can be made more toxic to weeds; or
3. the crop plant can be made more resistant to toxins produced by the weeds.

None of these alternatives has been studied extensively. The principle of modifying plant architecture is well established in the case of other useful agronomic traits, but is less exploited for weed control than might be expected. Sen (1988) "wonders why one cannot find varieties capable of resisting or suppressing weeds". Tanner et al. (1966) noted that, among small grains, varieties with upright leaves were lower yielding in the presence of weeds, higher in their absence, and that weeds were much more profuse among upright leaves than among more horizontal ones. Blackshaw et al. (1981) found the semidwarf wheat cultivar (cv.) Norquay to lose much more yield in the presence of green foxtail (*Seteria viridis*) than did two cultivars of normal height; the semidwarf wheat was shorter than foxtail throughout the growing season. Sweet et al. (1974) examined potato (*Solanum tuberosum*) cultivars Green Mountain and Katadhin. Green Mountain, a rapid starter, intercepted 60-70% of available light and was nearly free of weeds, while Katadhin intercepted very little and was severely infested with redroot pigweed (*Amaranthus retroflexus*) and lambsquarters (*Chenopodium album*). There appeared to be no interaction between roots of weed and potato, suggesting that in this case canopy architecture was critical. Forcella (1990) selected a line from a soybean (*Glycine max*) cross for rapid early leaf expansion and found that it produced 29% more than the parent variety under conditions of "intense weed competition".

Increasing the allelopathic potential of the crop plant has been the subject of limited study. Putnam and Duke (1974) found particular varieties of cucumber (*Cucumis sativum*) seed which suppressed germination of several other species more strongly. Seedlings of these varieties established more successfully in the presence of proso millet (*Panicum miliaceum*) (Lockerman and Putnam 1979). Fay and Duke (1977) examined 3000 accessions of oat (*Avena sativa*) for production of scopoletin, a coumarin, by seedlings; four accessions which produced markedly more were identified. Seedlings of one of these injured wild mustard (*Brassica kaber*) significantly more than a standard variety when grown together in pots of sand. Massantini et al. (1977) surveyed 141 soybean lines for allelopathic effect on the weeds *Helminthia echioides* and *Alopecuris myosuroides*. Soybean and weed seedlings were potted together in sand under conditions judged to minimize competition for light and nutrients. *Helminthia* was strongly inhibited by two soybean varieties; the weed seedlings showed signs of necrosis. *Alopecuris* was not significantly inhibited by any soybean variety. Both weeds were stimulated by one soybean variety. Alsaadawi et al. (1986) trapped root exudate from live plants of 100 sorghum (*Sorghum bicolor*) varieties and tested its effect on germination and early growth of *Amaranthus retroflexus*. Exudate from most varieties inhibited *Amaranthus* to some extent; significant variation existed among them. Decaying sorghum residues were more toxic and also showed variable toxicity among different sorghum lines.

The chief disadvantage of this approach is that plants other than weeds may be affected by increased crop toxicity. Carryover of phenolic compounds to the next crop would increase, possibly affecting it especially in low-tillage situations (Barnes et al. 1986; Purvis and Jones, 1990). Huber and Abney (1986) noted a soybean cultivar of increased sensitivity to soybean residues, probably due to allelopathy. Cases of reduced yield in no-till situations are common with some crops. It is possible that the crops and varieties best suited to no-till in terms of their own allelopathic potential may prove to be most toxic to following crops.

Livestock or humans eating the crop might also be affected by increased levels of allelochemicals. Livestock poisoning from high-coumarin varieties of sweet clover (*Melilotus alba*) was once common. Celery contains several linear furanocoumarins which cross-link DNA and cause increased cancer risk and photodermatitis. Many accessions contain more than the levels which cause photodermatitis (Trumble et al., 1990). Most vegetables have been bred for milder flavours than their wild progenitors and it seems likely that they therefore suffer increased interference from weeds.

Increasing production of allelochemicals also involves an energy cost to the plant. It is difficult to estimate the size of this cost. In cotton (*Gossipium hirsutum*), seed oil percentage drops as tannins and terpenoids in the apex increase (Hanny et al., 1978), and a negative correlation was found between gossypol content and cotton yield among primitive cotton races (Dilday and Shaver, 1980). Baldwin (1991) estimates that a 1% increase in nicotine content in tobacco (*Nicotiana tabacum*) leaves gives a reduction of half a million seeds per plant (cited by Fritz, 1992).

The third approach, that of increasing crop resistance to allelopathic chemicals produced by weeds, has not been approached systematically. Waller (1989) has recently suggested: "1. Recombinant DNA techniques. . . will be used in manipulating the DNA from crop plants to modify them for resistance to microorganisms, weeds and insects. 2. Resistance to allelopathic compounds will be selected from wild types of crop plants which have been grown in the presence of important weed species. 3. Screening of crop varieties and cultivars will be expanded to select those with the most number of allelopathic compounds or resistance to allelochemicals, or both." Yang (1982), referring to replant problems in asparagus (*Asparagus officinalis*), noted: "Preliminary observations indicate that some selections obtained from inbreeding cv. 'Mary Washington' grow well and are productive in old asparagus fields. This suggests that breeding autotoxin-resistant lines of asparagus is possible." Balke et al. (1987) suggest that "selection of crop varieties with higher levels of glucosyltransferases [which conjugate phenolic compounds] could lead to crops more resistant to allelochemicals". Nagarajan et al. (1992) note "a possibility of reducing interference from weeds by making crop plants resistant to allelochemicals". Recently some research has addressed the possibility of variation among cultivars in response to crop residues (Hicks et al., 1989). This approach has not otherwise been previously suggested, to my knowledge, in the literature.

In several cases certain cultivars of crops have been shown to be more resistant than others to interference by weeds (Sweet et al., 1974; Dilday et al., 1990; Reeves and Brooke, 1977; Peters and Zam, 1981), but in only one case were competitive and allelopathic factors distinguished. In this case, Sweet et al. (1974) suggested that early establishment and aggressive competition of potato varieties with weeds were of paramount importance. Data from Hicks et al. (1989) show different tolerances in two cultivars of cotton to wheat straw residue. This almost certainly indicates different responses to allelopathic chemicals. Variation between species clearly exists (Altieri and Doll, 1978; Wang et al., 1967).

The disadvantages of selecting resistant varieties are not known, but it is possible that more efficient breakdown of foreign allelochemicals might interfere with the production of these essential compounds within the plant. Consequently, the plant might be impaired in phytoalexin response, or in production of such important compounds as lignin, flavouring or colouring flavonoids.

A plant could become more resistant to allelochemicals by a variety of routes:

1. reduced entry of the allelochemical;
2. increased glycosylation of the allelochemical to decrease hydrophobicity;
3. increased hydroxylation or decarboxylation;
4. faster compartmentalization into vacuoles; or
5. faster polymerisation into lignin.

Selection for greater resistance to allelopathic weed extracts, or chemicals involved in allelopathy, might lead to changes in any of these. Current knowledge does not allow us to predict which responses might be most likely. Introduction of new genes from other organisms is more controlled in terms of genetic change, although expression of foreign genes within a plant may also show unpredictable patterns (van der Krol et al., 1990; Napoli et al., 1990; Elkind et al., 1990). Both these approaches, however, since they employ well-known and simple selective techniques and avoid the disadvantages of increasing the toxicity of the plant and the energy costs of increased synthesis of allelochemicals, seem quite promising.

1.6 Identification of model crop/weed system

Flax was selected as a model crop system. It has several advantages both agronomically and in the laboratory. It is highly susceptible to interference by such weeds as wild oats, much more so than are wheat, canola (*Brassica campestris* or *napus*), or barley (O'Donovan and Sharma, 1983). Its limited leaf area index is very likely to contribute to this sensitivity, as it is relatively unable to suppress weeds by shading. However, growth chamber trials showed that flax is more susceptible to several phenolic acids and coumarins than are wheat, barley or oats (Section 3.1.1). It is, therefore, a species which might benefit more than others from increased resistance to toxins. It is easy to grow, large enough as a seedling for different effects of allelopathic compounds to be observed easily, and is consistent in response.

It can readily be grown in tissue culture as callus, leafy callus, or suspended cells (Murray et al., 1977).

It is transformable by *Agrobacterium tumefaciens*, and regeneration of transformed material has been reported (Jordan and McHughen 1988; Basiran et al. 1987).

Flax is true-breeding, with above 98% self-fertilization (Kenaschuk, 1975), and has no near relatives in North America (Kenaschuk, 1975). The possibility of an introduced gene escaping into related species, should the transformed plant be grown in the field, is therefore minimal.

Flax is a crop of major importance in most areas of the world and the third most important oilseed crop in Canada (Agriculture Canada, 1991), which is the world's largest producer and exporter of oilseed flax (Wilkins, 1988). Formerly it was much more widely grown in Canada than at present, and among the reasons cited for its discontinuance was that European weeds had caught up with the crop (Wilkins, 1988). Therefore, it may be a crop which is more sharply limited in distribution by allelopathy than most other crops.

The effects of allelochemicals on wheat, barley and oats were examined more briefly, to find whether allelopathic effects exist and how they ranked in importance relative to those seen in flax, and to find whether flax could be considered unusually sensitive to allelochemicals or not.

Tobacco was also used in the project, because of the ease of transformation and of regenerating transformed plants in this species (Draper et al., 1988). The pattern of expression of the *mas* promoter used has been documented in tobacco (Langridge et al., 1989). Work on tobacco intensified when regeneration of transformed flax proved difficult.

Among weeds, wild oats and Canada thistle (*Circium arvense*) were used most. These are among Canada's most common weeds (Moore, 1975; Alberta Weed Survey, J. O'Donovan, unpublished data, 1988) and both figure on the list of the world's worst weeds (Holm et al., 1977). Both are highly injurious to wheat and flax and appear to be allelopathic (Schumacher et al., 1983; Pérez and Ormeño-Nuñez, 1991; Tinnin and Muller, 1972; Bendall, 1975; Wilson, 1981), although no numerical estimate of their allelopathic components has been made.

Yield losses to wild oats and Canada thistle are common. Dew (1972) and Dew and Keys (1976) constructed indices for yield loss from known numbers of wild oats in barley, wheat, canola and flax. At 50 wild oats/m^2, these suggest losses of 16% for barley, 24% for wheat, 23% for canola and 42% for flax, under western prairie conditions. O'Sullivan and Kossatz (1984) used the same approach to make indices for Canada thistle. At 50 thistles/m^2, these indices suggest losses of 54% for barley, 79% for wheat, 75% for canola and 100% for flax. These indices are in part extrapolated from Dew's, on the apparent assumption that crops respond to all weeds in the same way, and have not been verified.

Tinnin and Muller (1971) suggested that, in chaparral/grassland mosaics in California, leachates from wild oats were important in maintaining the herb pattern. The grassland portion is strongly dominated by wild oats. They found (1972) that wild oat straw applied by several means inhibited germination and shoot extension of several tested species. *Centauea militensis*, a species excluded from wild-oat-dominated grassland but present nearby in the chaparral and border zones, was strongly inhibited. Flax and wild oats were less inhibited. Schumacher et al. (1983) found that exudates of wild oat seedlings reduced shoot and root dry weight of wheat seedlings by up to 40%.

Oat and wild oat seedlings are known to exude scopoletin (Martin and Rademacher, 1960; Fay and Duke 1977). Wild oats is closely related to domestic oats, and they may be considered the same biological species (Baum 1968). The phenolic components of mature wild oats have not been quantitated, but several phenolic acids (*p*-

coumaric, ferulic, and vanillic acids) and scopoletin have been identified in wild oat hay (Tinnin and Muller 1972). Root exudate from wild oats with 1-4 leaves contains vanillic and *p*-hydroxybenzoic acids, scopoletin, coumarin and possibly umbelliferone (Schumacher et al., 1983; Pérez and Ormeño-Nuñez, 1991). The amount of scopoletin recovered was too small to injure most plants, but these wild oat seedlings were grown under conditions which would minimize phenolic production. Domestic oat residue was found to contain the equivalent of 26 kg/ha of various phenolic acids (Guenzi and McCalla, 1966). This is much less than many other domestic species, such as corn (81 kg/ha) or sorghum (100 kg/ha), but some more toxic components such as scopoletin were not estimated.

Canada thistle has been shown to be highly allelopathic. Bendall (1975) found that water extract of *C. arvense*, or its dry residues, severely reduced radicle extension of several thistle and other species, including barley, perennial ryegrass (*Lolium perenne*), and subterranean clover (*Trifolium subterraneum*). Wilson (1981) found similar effects on wheat, alfalfa (*Medicago sativa*) and sugarbeet (*Beta vulgaris*). Stachon and Zimdahl (1980) found species diversity in the field to be greatly reduced in the vicinity of Canada thistles and that barley and several weeds were injured by thistle extracts.

The phenolic contents of Canada thistle have not been previously examined. Paper chromatography of a water extract of Canada thistle top (made by soaking Canada thistle tops overnight in water to cover, at approximately 4% fresh weight per volume) showed the presence of at least ten different fluorescent (probably phenolic) compounds under ultraviolet light. Vanillic acid and *p*-hydroxybenzoic acid were identified; the other compounds were not any of the common phenolic acids or coumarins. The total concentration of flourescent compounds in the extract used was estimated at about 10 mM (Ray, unpublished results). Many phenolic compounds at 4 mM severely stunt flax and wheat seedling growth.

I wished to know whether action of these compounds in combination, as occurs in nature, was less than additive, additive, or synergistic. The literature on this point is somewhat contradictory, and has been complicated by confusion in use of terms and the non-linear response to allelochemicals of many plants. Here, additivity means that the effects of mixtures of pure compounds is equal to the averaged effects of the pure compounds used separately, synergy means that the effects of mixtures are greater than expected from averaging the effects of pure compounds, and less than additive means that the effects of mixtures are less than expected from averaging the results of pure compounds. Non-linear response to allelochemical dosage is evident in Figures 11 and 12. Because of this non-linearity, it is necessary when combining phenolic acids to keep the total concentration constant. Therefore the effects of actual mixed doses are compared to the average of the effects of the components used singly, rather than to summed effects.

Rasmussen and Einhellig (Einhellig and Rasmussen, 1978; Rasmussen and Einhellig, 1977, 1979) studied the effects of combinations of phenolic acids on grain sorghum and radish (*Raphanus sativum*) germination and early growth. These papers claim to show synergy but their results are indistinguishable from additivity (as also noted by Duke et al., 1983). Duke et al. (1983) examined lettuce (*Lactuca sativa*) germination, finding no significant interaction between ferulic and *p*-coumaric acid (i.e. additivity) and some antagonism between either of these and caffeic acid, suggesting competitive inhibition. The results of Gerig and Blum (1991) on leaf area expansion of cucumber seedlings found additivity between ferulic, *p*-coumaric and vanillic acids and antagonism between these and *p*-hydroxybenzoic acid. Lyu et al. (1990), studying phosphorus uptake in cucumber seedlings, found additivity between ferulic, *p*-coumaric and vanillic acid in nearly all combinations. The majority of interactions betweeen phenolic acids of similar chemical structure appear to be additive.

Many crops are themselves allelopathic. Allelopathy has not been attributed to flax, although it contains several hydroxycinnamic acid derivatives and flavones (Ibrahim and Shaw, 1970; Liau and Ibrahim, 1973). Barley, sometimes used as a "smother crop", produces allelopathic leachate from seed or root. This leachate injures chickweed (*Stellaria media*) and shepherd's purse (*Capsella bursa-pastoris*), but not wheat (Overland, 1966). Liu and Lovett (1990) found that 6 day old barley seedlings released enough of the alkaloids hordenine and gramine to reduce germination and growth of white mustard (*Sinapis alba*). *Brassica* species have long been considered phytotoxic. Mason-Sedun et al. (1986) found significant effects of residue from six *Brassica* species on wheat seedlings, and identified variation between cultivars of *B. juncea*. Vera et al. (1987) found that fall-sown canola seedlings ploughed down in spring reduced yields of several following crops; flax by 65%, *B. campestris* by 53%, barley by 23%, wheat by 17%, and oats unaffected. Jiménez-Osornio and Gliessman (1987) showed *B. campestris* and *B. oleracea* to be allelopathic to a variety of indicator species. Wheat may be phytotoxic also. Lodhi et al. (1987) found inhibition of cotton and wheat seedlings by wheat mulch and extract. Pérez (1990) found that the principal hydroxamic acid of wheat, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, and its principal breakdown product inhibited germination and root extension of wild and domestic oats. Wild oats was much more affected than domestic oats.

Guenzi et al. (1967) examined the effects of wheat, oats, corn and sorghum residue on wheat seedlings. Oats and sorghum strongly inhibited wheat root and shoot development; sorghum residues remained toxic for 16-22 weeks. Putnam and DeFrank (1983) compared effects of mulches of several crops on several vegetable and weed species. Of wheat, rye (*Secale cereale*), oats and barley, wheat was most inhibitory and rye least. However, rye reduced following crops most, while barley stimulated them, under no till conditions. Pérez and Ormeño-Nuñez (1991) found that, while both rye and wheat have high hydroxamic acid levels in their leaves, only rye exudes them from its roots and inhibits wild oat growth as a result. The relative allelopathy of wheat and rye would therefore depend on whether live crops or residue was being considered.

Further research may clarify and detail some of the mutual effects of crops and crop residues with weeds. It must be emphasized that these interactions are complex and delicately balanced.

1.7 Identification of variation in response to allelopathic chemicals

I had two main reasons for seeking to identify significant variation in response to allelopathic chemicals, in a crop species. First, the simplest and most practised way to alter any genetic trait is to select for its presence or absence. The existence of variation in the trait being considered is a necessary precondition. Once identified, suitable strains can be used for crossing or selection. Second, that a phenomenon can be manipulated testifies to the existence of the phenomenon. Showing the existence of significant variation in response to allelochemicals adds to the evidence for the existence of significant allelopathy.

No species had been examined for variation in response to allelochemicals when this project began. Since then, Waller (1989) has noted the possibility, and A. Kilvert has shown the existence of significant variation among alfalfa varieties in response to several coumarins and phenolic acids (M. Sc. Thesis, University of Alberta, 1991). Research has indicated the existence of variable response to weed interference among cultivars of several species, such as tall fescue (*Festuca arundinacea*) (Peters and Zam, 1981), soybean (McWhorter and Hartwig, 1972), rice (*Oryza* spp.) (Dilday et al., 1990) and wheat (Reeves and Brooke, 1977). In no case were allelopathic and competitive aspects of interference distinguished, but these data are suggestive of allelopathic effects. Recently, varietal variation in response to residues (presumably corresponding to variation in response to allelopathic compounds in residues) has been found among cotton cultivars (Hicks et al., 1989).

The response of several cultivars of *Linum usitatissimum* and some related *Linum* species, to *p*-coumaric acid, was examined. The response of several cultivars of barley to wild oat shoot extract, *p*-coumaric acid, and scopoletin was also examined. If identifiable, differences in such responses among varieties could be exploited to produce crop plants of increased weed tolerance if allelopathy is a significant component of interference.

1.8 Identification of suitable genes for introduction into crop species

Organisms which consume dead or living plants are reasonable places to look for genes which detoxify plant phenolic compounds. This rather generous allotment of possibilities was narrowed by screening for good growth of microorganisms on medium containing high levels of ferulic acid or coumarin, and by looking for well-known detoxification enzymes for which genes had previously been cloned, from any organism. The work done with microorganisms will not be described in detail, but will be briefly summarized in this section.

Among laboratory organisms, baker's yeast (*Saccharomyces cerevisiae*) displayed good resistance to phenolic compounds. Wash from Canada thistle root gave a natural flora of resistant microorganisms. Among this group, one bacterial species stood out. It was identified by Dr. L. Goatcher (Alberta Environmental Centre, Vegreville) as *Pseudomonas fluorescens* biovar V. Yeast and *Pseudomonas* were therefore investigated as potential donors of genes conferring resistance. Some other potential donors are also discussed. Among previously cloned genes expected to confer resistance, two mammalian P450 genes were examined and chosen for transformation into flax and tobacco.

1.8.1 *Pseudomonas* species

The great variety of organic molecules that various species of *Pseudomonas* can degrade has led to much research, principally for bioremediation. The bulk of its abilities arise from the *meta*- and *ortho*-cleavage of phenolic rings (Burlage et al., 1989). The TOL plasmid pWWO has a broad spectrum specificity for substituted benzoic acids such as chlorobenzoates (Ramos et al., 1986), using hydroxylation followed by ring cleavage in the *meta* pathway (Harayama et al., 1987). However, plasmid-free strains can grow with benzoate as the sole carbon source, using the *ortho* pathway of degradation (Duetz et al., 1991). Another plasmid, the Sal plasmid, encodes enzymes which degrade benzoic acids and salicylic acid; in this case benzoates induce and are degraded by the *ortho* pathway while salicilate induces the *meta* pathway (Chakrabarty, 1972).

Very little work appears to have been done with cinnamate derivatives or coumarins in *Pseudomonas*, and relatively little with the chromosomally-encoded *ortho* pathway, all attention being drawn by the large plasmids conferring such exotic abilities as degradation of toluene or halogenated aromatics. Vanillate is degraded beginning with a chromosomally-encoded demethylase (Brunel and Davison, 1988); benzoate and several substituted benzoates by either the *ortho* or *meta* pathway depending on circumstance and strain (Figure 2). *P. acidovorans* converts ferulate to vanillate, which is then reportedly degraded by the *meta* pathway in this species and by the *ortho* pathway in *P. fluorescens* (Dagley, 1971). It seems likely that *Pseudomonas* either can degrade cinnamates directly by the same enzymes as benzoates or by first converting them to benzoates; both possibilities may coexist. Coumarins may possibly be re-converted to cinnamic acids before degradation by the *meta* or *ortho* pathways, or with the use of some other preliminary steps (Kielich, 1976).

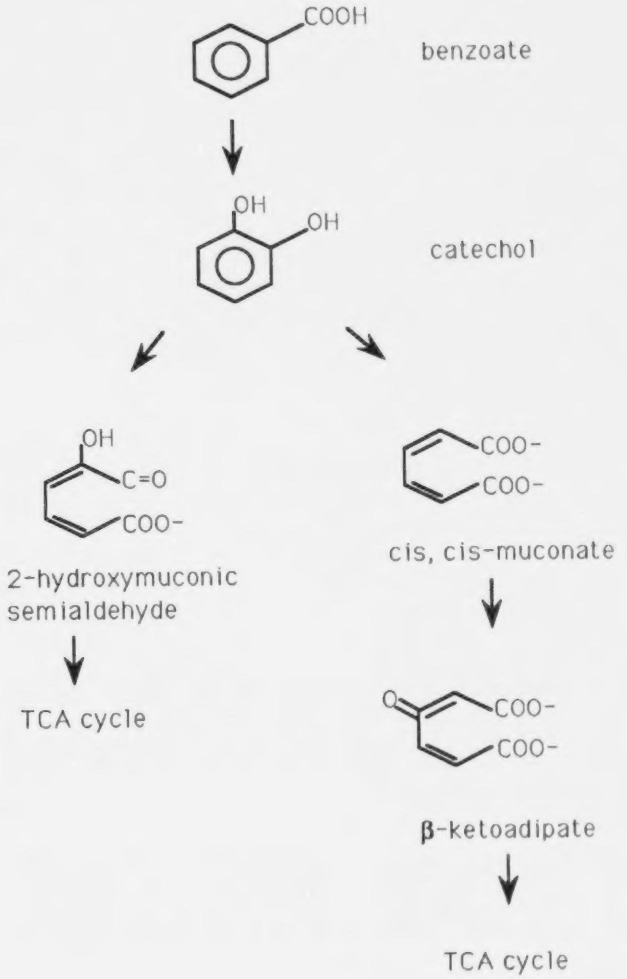

COOH
benzoate
OH
OH
catechol
OH
C=O
COO-
2-hydroxymuconic semialdehyde
TCA cycle
COO-
COO-
cis, cis-muconate
O
COO-
COO-
β-ketoadipate
TCA cycle

Figure 2. Degradation of benzoate by *meta* (left) and *ortho* (right) pathways of *Pseudomonas* (Burlage et al, 1989).

Following the identification of *P. fluorescens* in soil washed off Canada thistle root, the growth of *P. putida* with and without the TOL plasmid, encoding the *meta* cleavage pathway, was examined. Both grew well at substantial concentrations of several phenolic compounds. The plasmid-free strain, however, grew marginally better on 40 mM ferulate and 20 and 30 mM *p*-coumarate; no other differences were noted (Ray, unpublished results). The *meta* pathway was therefore not needed to degrade these compounds in *P. putida.* Since no genes with obvious potential for transfer to plants were available, work with *Pseudomonas* was discontinued.

Field-isolated *Pseudomonas* inactivates phenolic acids and reduces their toxicity to wheat seedlings (Vaughan et al., 1983). Useful *Pseudomonas* genes may be isolated and transferred into plants at some time.

1.8.2 *Saccharomyces cerevisiae* and other yeast species

Many yeasts normally subsist on decaying (fermenting) plant material, where phenolic compounds may be present in large amounts. The effects of phenolic compounds on *S. cerevisiae* have been examined by Van Sumere et al. (1972) and Baranowski et al. (1980). Pathways of phenolic metabolism in yeasts are generally uncharacterized, however. *Saccharomyces cerevisiae* has been well characterized genetically in other regards, *S. cerevisiae* DNA libraries are available, and the molecular biology of this organism is well developed. *S. cerevisiae* has a minute genome among eukaryotes (2×10^7 base pairs) and can be maintained as a haploid or a diploid. These factors made this yeast an excellent potential source of resistance to phenolic compounds, if single genes for this trait could be found.

Two main lines of research using *S. cerevisiae* were undertaken. Four series of yeast mutants of increased sensitivity to phenolic acids and coumarins were developed. Cross-resistance patterns and complementation analysis showed very complex patterns, with a large number of complementation groups.

Libraries of wild type yeast DNA were searched for sequences conferring resistance to ferulic acid or coumarin, using multicopy plasmids transformed into wild type yeast. I hoped to find that overexpression of particular gene sequences would provide increased resistance to these compounds, as has been the case for resistance to other compounds in yeast (Rine et al., 1983; Leppert et al., 1990). The relevant DNA could then be subcloned and sequenced. At least two plasmids which conferred increased resistance to ferulic acid and coumarin were identified. These plasmids were characterized to the point of restriction mapping (Ray, unpublished results). The biochemical nature of this resistance has not been examined.

POF1 strains of yeast create phenolic off-flavours in beer by converting ferulic acid to a styrene derivative (Chen and Peppler, 1956). A single gene, POF1, was cloned and by disruption experiments found to be responsible for the decarboxylation (Meaden and Taylor, 1991). The POF1 product converts ferulate to 4-vinylguiacol, *p*-coumarate to 4-vinylphenol, and *t*-cinnamate to styrene (Figure 3). It does not act on caffeate or *p*-hydroxybenzoate (Goodey and Tubb, 1982). POF1 strains have increased resistance to *p*-coumarate and *t*-cinnamate (Hope, 1987).

No cross-hybridization of DNA between the POF1 plasmid and either of the two sequences isolated through overexpression was seen. *S. cerevisiae* therefore contains at least three different genes conferring resistance to some phenolic compounds. Complementation results among mutants of *S. cerevisiae* suggest that there are likely to be more.

Cytochrome P450 monooxygenases are involved in the elaboration of phenolic compounds in plants and their detoxification in animals. Several P450 genes occur in yeast species (Käppeli, 1986; Sariaslani, 1991); at least four have been cloned (Kalb

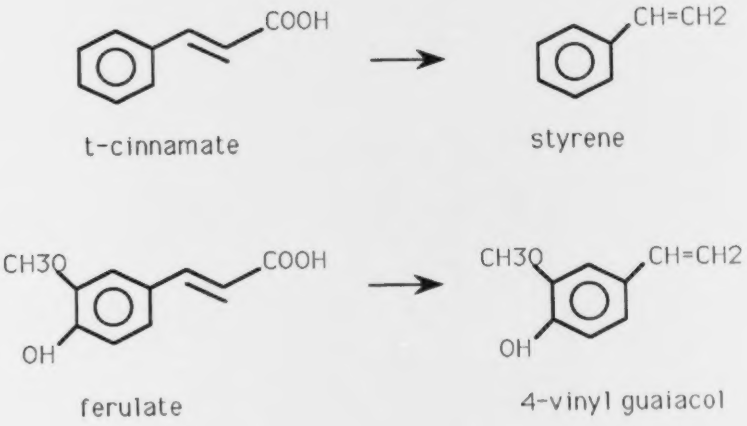

COOH
CH=CH2
t-cinnamate
styrene
CH3O
COOH
OH
CH3O
CH=CH2
OH
ferulate
4-vinyl guaiacol

Figure 3. Decarboxylation of phenolic acids by POF1.

et al., 1987; Sanglard et al., 1987; Seghezzi et al., 1991; Chen et al., 1988). Other fungi are known to contain P450 genes which detoxify phenolic compounds (Matthews and Van Etten, 1983; Miao et al., 1991). The chances of finding P450 gene involvement in yeast phenylpropanoid metabolism are probably good.

The yeasts appear to have several different methods of detoxifying phenolic compounds, by cleavage, decarboxylation and probably by hydroxylation and/or demethylation. Since most of the probable products are less toxic to plants than are the precursors, genes useful for transformation into plants may be isolated in the future. Time limitations, however, required that these lines of inquiry be dropped.

1.8.3 Cytochrome P450 monooxygenases

Among other detoxification genes, the superfamily of cytochrome P450s (mixed function oxidases) was of interest. Some genes in this group were reported to have some activity on coumarin or similar phenolic compounds (Wood and Conney, 1974), and two genes were selected for transformation into plants. Some background on the P450 superfamily is provided.

Cytochrome P450 monooxygenases (P450s) are a numerous family of mixed function oxidases with a sweeping range of oxidative capacities. Their many functions include detoxification of xenobiotics, drug metabolism, detoxification of herbicides, steroidogenesis, and synthesis of phenolic acids and flavonoids. Certain xenobiotics are activated rather than detoxified by them because of the formation of radical intermediates; benzo(a)pyrene is an example (Gonzalez, 1989).

P450 enzymes appear to be ubiquitous, having been found in vertebrates, insects, plants, fungi and bacteria. Every organism may benefit from the capacity to detoxify poisons taken in from the environment. It is reasonable that plants, over their evolutionary history, have elaborated more and more complex phenolic compounds to combat herbivores and pathogens, and that in plants P450 enzymes are chiefly involved in the synthesis of these compounds. Meanwhile their foes have had to develop methods of detoxifying the phenolic compounds they are obliged to take in, and in them P450 enzymes were first important in detoxification of xenobiotics, with roles in steroidogenesis probably developing later.

Prokaryotic P450 enzymes are found in the cytosol, as are eukaryotic forms identified in hepatic and adrenal mitochondria (Shayiq and Avadhani, 1989). All other eukaryotic forms are found on membranes and are usually isolated from microsomal fractions. The best studied source of P450 enzymes is the mammalian liver, in which several forms with narrow or broad range enzymatic capacities occur.

While overall P450 enzyme patterns are similar between related species, variation exists even within species. Often this is manifested as variation in rates of drug metabolism. Different strains of mice, for example, respond very differently to the same dose of coumarin (Wood and Conney, 1974; Lush and Andrews, 1978), while rats metabolize almost no coumarin (Raunio et al., 1988). Variation in drug metabolism and interactions in humans, a frequent medical problem, is strongly affected by allelic variation in P450 genes.

Thorough recent reviews of vertebrate P450 enzymes are those by Gonzalez (1989) and Nebert and Gonzalez (1987). Fungal P450s are reviewed by Käppeli (1986) and by Sariaslani (1991), bacterial P450s by Sligar and Murray (1986) and by Sariaslani (1991). Plant P450s are reviewed by Higashi (1985) and Donaldson and Luster (1991) and in herbicide-resistance aspects by Jones (1991).

1.8.3.1 Physical characteristics of P450 enzymes

Cytochromes P450 were identified as a new class of cytochrome with a Soret peak at 450 nm when bound to CO (Garfinkel, 1958; Klingenberg, 1958). This class was found to be a heme-containing protein (Omura and Sato, 1964) with cysteine residues critical for binding the heme (Shimizu et al., 1988; Poulos, 1988). Eukaryotic forms are almost all membrane bound, usually sitting on the cytosolic side of microsomal membranes. They require signal recognition particles (Sakaguchi et al., 1984) and a 20 amino acid leader sequence (Monier et al., 1986) for insertion into the membrane. The leader sequence is not cleaved and anchors the protein to the membrane rather than pulling it through (Sakaguchi et al., 1987). Most P450 enzymes are in the 45-57 kD range.

The most frequent monooxygenation performed by P450s is:

$$R + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O$$

Electrons are supplied by NADPH-cytochrome-P450-oxidoreductase (reductase) in most systems, the reductase receiving them from the electron transport chain. The catalytic sequence is as follows:

1. substrate binds to P450 enzyme;
2. NADPH reduces flavin prosthetic groups of reductase;
3. one electron transferred from reductase to P450 enzyme;
4. O_2 binds to P450 enzyme;
5. second electron transferred from reductase (sometimes from cytochrome *b5* or other molecules) to P450 enzyme;
6. O=O cleaved; distal O incorporated with 2 H^+ into water;
7. proximal O bound to substrate;
8. oxidized substrate dissociates from P450 enzyme

(Ortiz de Montellano, 1986).

The most common result of a P450 enzyme reaction is a hydroxyl group, but many other possibilities exist; they can dealkylate, deaminate, demethylate, *N*-oxidize, dehalogenate or form sulfoxides (Nebert and Gonzalez, 1987). Insertion of the O atom may produce unstable ion intermediates which participate immediately in further reactions. The reaction may repeat itself so that P450 enzyme can act as a dioxygenase.

Cytochrome P450 enzymes appear to be highly conserved in tertiary structure, particularly in the core region. To date only one P450 enzyme, the camphor-binding P450cam of *Pseudomonas putida* (P450CIA1) has been crystallized and studied at a high level of resolution (Poulos et al., 1985, 1986, 1987; Poulos, 1988, 1991). Although lacking a membrane-anchoring segment, the soluble bacterial P450 is otherwise similar to membrane-bound eukaryotic P450 enzymes (Nelson and Strobel, 1989; Poulos, 1991).

The following description of P450 enzyme structure and activity is based on P450cam, with additional information from sequence comparisons and site-specific mutagenesis. P450 enzymes are composed of a single polypeptide chain. They form a compact pyrimidal shape, with the heme group almost exactly centered. The iron ligands include the four nitrogens of the pyrrole ring common to all hemes. The fifth ligand is a cysteine residue (Cys 357 in *P. putida*), highly conserved among P450 enzymes (Shimuzu et al., 1988; Poulos, 1988). At the sixth ligand, opposite the cysteine residue, the reaction is catalysed by a mechanism that remains unclear (Poulos, 1988).

A deep, very narrow channel approaches the central heme. This substrate channel is hydrophobic and evidently flexible, as some substrates are much larger than the channel. Camphor appears to hydrogen bond to Tyr 96 within the channel; Tyr 96 is not highly conserved and it is probable that changes in this area provide substrate specificity. The O_2 molecule is held in a pocket adjacent to camphor and heme (Poulos et al., 1987; Poulos, 1988).

The iron atom central to the heme is maximally spin paired in the absence of substrate (low-spin state). In most but not all P450 enzymes, substrate binding causes a shift to the high-spin state. The substrate is placed directly above the heme. The sulphur atom of the invariant cysteine coordinates with the iron atom and is almost certainly critical in the breaking and making of bonds (Poulos, 1988). Electrons are most commonly supplied to P450 enzymes by a flavoprotein P450 reductase. Poulos suggests that electrons may enter the catalytic site via a hydrogen bonding path (Poulos et al., 1987).

The substrate recognition and binding sites of P450 enzymes vary much more than the catalytic features, as would be expected. Single residue changes may be critical. Site-specific mutagenesis of the 11 residues differing between testosterone$_{15\alpha}$ hydroxylase and coumarin hydroxylase (coh) showed that 3 residues were critical for activity and that the single residue at 209, phenylalanine in P450coh and leucine in testosterone$_{15\alpha}$ hydroxylase, sufficed to change the substrate specificity completely (Lindberg and Negishi, 1989). A series of site-specific mutations at residue 209 changed its spin state according to size and hydrophilicity of the residue (Iwasaki et al., 1991). Phenylalanine is believed to sit close to the sixth ligand of the heme (Iwasaki et al., 1991) and recognise the keto group of coumarin, its critical feature (Juvonen et al., 1991). A single amino acid change, glycine to aspartate at residue 310 in *Saccharomyces cerevisiae* lanosterol demethylase, abolishes the binding of lanosterol (Sariaslani, 1991).

Numbers of known P450 genes and enzymes have been increasing extremely rapidly. In a large number of species multiple forms of P450 enzyme exist. To date 34 forms have been identified in the rat (Nebert and Nelson, 1991), and a total of 60-200 P450 genes per mammalian species has been suggested.

The large size of the P450 superfamily necessitates an orderly system of nomenclature. Current nomenclature follows Nebert (Nebert et al., 1989; Nebert and Nelson, 1991), whose system is as follows: P450 proteins are grouped in families, designated by Roman numerals, in which all members share at least 40% DNA sequence identity; subfamilies, designated by capital letters, sharing at least 59% sequence identity; individual proteins are designated with Arabic numerals. Within a species, sequences more than 97% identical are assumed to be allelic unless proven otherwise. Sequences from different species are matched as well as possible and similarly named. Genes are named in parallel fashion to proteins, with *CYP* (*Cyp* in mouse) replacing P450 and Arabic numerals replacing Roman. Thus the gene encoding P450IA1 is *Cyp*1A1 and that for coumarin hydroxylase is *Cyp*2A5 (Puga and Nebert, 1990).

P450 genes follow no consistent pattern of induction or structure. Among the hundred-plus currently cloned (Puga and Nebert, 1990), cases have been found in which proteins extremely similar in amino acid sequence differ so markedly in their induction patterns as to be formerly placed in different families, or extremely similar proteins have very different substrates, or dissimilar proteins act on the same substrate. Cases are also known of alternative splicing (Goldfarb 1990), multiple transcription start sites, pseudogenes and apparent partial gene conversion (Gonzalez, 1989).

The bulk of P450 gene regulation appeares to be transcriptional, but some important exceptions have appeared. A number of compounds (phenobarbital, 3-methylcholanthrene, dexamethasone) induce an astonishing array of different P450 enzymes, sometimes in organisms widely dissimilar in life habits and expected exposure to the compounds. For example, phenobarbital appears to induce P450 enzymes in mice and other mammals (Gonzalez, 1989), plants (Reichhart et al., 1980), and *Bacillus megabacterium* (Murakami et al., 1987). The apparent induction may in fact be due to stabilization of the enzyme by phenobarbitol. Other substrates may also stabilize P450 enzymes.

1.8.3.2 Selection of P450 genes for transformation into plants

The very incomplete knowledge of substrate ranges for P450 enzymes made it difficult to select the P450 gene with the best chance of performing the desired function in plants, namely, the detoxification of allelochemicals. Isolation of P450 enzymes and genes has moved very rapidly, while defining substrate ranges of the individual enzymes has lagged.

The isolation and characterization of the P450IA1 and P450IIA5 genes are reviewed, and some relevant plant P450 genes are discussed below.

1.8.3.2.1 P450IA1

P450IA1 (P1) enzyme of mouse was isolated and cDNA from the encoding gene *Cyp*1A1 was sequenced by Kimura et al. (1984). Its specificity is broad, but it is most frequently studied in response to benzo(a)pyrene or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin), both of which it activates to more potent carcinogens. P450IA1 may be the best studied of eukaryotic P450 genes and has been expressed in at least two heterologous systems (Kimura et al., 1984; Nakamura et al., 1983; Gonzalez and Nebert, 1985; Cullin and Pompon, 1988). It has demonstrated although minor activity towards coumarin and ethoxycoumarin (Peters et al., 1991). Various assays to measure its activity were available (Oeda et al., 1985; Nebert and Gelboin, 1968).

1.8.3.2.2 P450IIA5

P450IIA5 (coh), encoded by *Cyp2A5*, is the best studied of P450 enzymes in relation to coumarin compounds. It is induced by phenobarbital and pyrozole (Kojo et al., 1991) and presumably by coumarin, but I have not been able to locate any statement on this topic. Coumarin is reported to stabilize the enzyme during isolation (Juvonen et al., 1988).

Recently, the mouse form of the gene was found to have been cloned under a different name and presumed function. In studying $P450_{15\alpha}$, which is expressed at high levels in female liver and male kidney of mouse, Squires and Negishi (1986) found and cloned two cDNAs, forms I and II (Burkhart et al., 1985; Squires and Negishi, 1988; Lindberg et al., 1989). Form I is expressed constitutively at much higher levels, accounts for the sexual dimorphism in expression (Squires and Negishi, 1988), and hydroxylates testosterone and other 3-keto Δ^4 steroids efficiently (Lindberg et al., 1989). Form II does not perform this reaction, but is nearly identical in sequence, varying at only 11 of 494 amino acid residues. Unexpectedly, form II was found to hydroxylate coumarin and its derivatives efficiently and was identified as coumarin hydroxylase (P450IIA5) (Negishi et al., 1989). P450IIA5 is now relatively well characterized (Lindberg and Negishi, 1989; Negishi et al., 1989; Juvonen et al., 1991) and has been expressed in SV40-transformed COS monkey kidney cells (Negishi et al., 1989; Lindberg and Negishi, 1989) and in yeast (Iwasaki et al., 1991). Several assays for coumarin hydroxylation are available (Aitio, 1978; Kapitulnik et al., 1977; Kaipanen et al., 1985).

1.8.4 P450 genes of plants

Research on plant P450 enzymes has lagged behind that on other systems. Two plant P450 genes have been cloned and sequenced (Bozak et al., 1990; Vetter et al., 1992). Several proteins have been isolated.

In plants, P450 enzymes are involved at several points in the synthesis of phenolic compounds. Unlike many mammalian P450 enzymes, they are so far highly selective as to substrate. *T*-cinnamate 4-hydroxylase and ferulate 5-hydroxylase, both P450 enzymes (Gabriac et al., 1991; Grand, 1984; Higashi, 1985), are essential to most, possibly all, vascular plants. Cinnamate hydroxylase converts *t*-cinnamic acid to *p*-coumaric acid (Figure 1) precursor to coumarins, flavonoids, lignin and a host of other products. This activity is induced by a variety of factors: ethanol, herbicides, manganese and phenobarbital (Reichhart et al., 1980) and is also regulated by product and substrate concentrations (Loake et al., 1991). Ferulate hydroxylase converts ferulic acid to sinapic acid, a precursor to lignin (Figure 1). It has been proposed that control over lignin synthesis may be exerted via this enzyme (Grand, 1984). Lignin is among the principal mechanical defenses of land plants and the chief contributor to rigidity of woody plants, while flavonoids and other phenolic compounds constitute the chief chemical defenses of plants against herbivores and pathogens.

Several further P450 enzymes have been found interconverting and elaborating flavonoids and indole alkaloids. Unlike those already discussed, they are not universal and may be confined to quite small genera. They are fairly substrate specific. P450 enzymes have also been implicated in resistance to herbicides such as monuron and aldrin (O'Keefe et al., 1987) and dicamba (Comai and Stalker, 1986). Other herbicides are metabolized by resistant plants to the same compounds produced in mammalian liver by P450 enzymes, suggesting that they also may be inactivated by these enzymes (Comai and Stalker, 1986). An allene oxide synthase in the jasmonic acid pathway has also been identified as a P450 enzyme (Song and Brash, 1991), as have two lauric acid hydroxylases (Zimmerlin et al., 1992). Vetter et al. (1992) found evidence for at least eight P450 enzymes in periwinkle (*Catharanthus roseus*) cultures. P450 enzymes are also implicated in synthesis of gibberellins, terpenes and sterols (Donaldson and Luster, 1991). Plants may eventually be found to have as many of these enzymes per species as mammals.

1.9 Transformation of P450 genes into plants

P450 genes *Cyp*1A1 and *Cyp*2A5 were transformed into tobacco and flax by means of the *Agrobacterium tumefaciens* Ti plasmid, which has the unusual ability to integrate part of itself into the genome of a plant. An extensively modified Ti plasmid with cloning sites, polyadenylation signals, and marker genes to facilitate selection was used. The mouse genes were placed in this plasmid so that they, with marker genes for kanamycin resistance and for the production of a light-emitting protein, were integrated into the plant genome in a form expected to be expressed.

Agrobacterium biology and its use in plant genetic engineering has been thoroughly studied and reviewed (Herrera-Estrella and Simpson, 1988; Binns and Thomashow, 1988; Hooykaas, 1989; Zambryski et al., 1989; Draper et al., 1988; Winans, 1992). *A. tumefaciens* is a soil bacterium which causes crown gall disease of many dicotyledonous plants. The crown gall is a swollen outgrowth of callus-like cells which have been transformed by the large Ti plasmid, of which a part, between short specific left and right border sequences, enters the plant cell and integrates more or less at random into the plant nuclear DNA. The wild-type inserted sequence carries genes for the synthesis of opines, which the bacterium can metabolize but the plant cannot, and for plant hormones IAA and cytokinin, so balanced as to cause rapid, disorganized cell growth and division of plant cells containing food for bacteria. Thus the bacterium engineers plant metabolism for its own use. Now it has itself been subverted by the replacement of opine and phytohormone genes by others of interest to plant biologists.

A large number of virulence (*vir*) factors are required for the transformation; some are on the Ti plasmid, some on the bacterial chromosome. For genetic engineering, the plasmid functions are usually split into two parts, a helper plasmid encoding necessary *trans*-acting *vir* functions, and a small vector plasmid with little of the original Ti plamid except its border sequences. This plasmid usually carries suitable promoters, cloning sites, and selectable markers. After insertion of a gene of interest, the plasmid is transformed into an *E. coli* strain which, by the aid of a helper plasmid, transconjugates it into an *Agrobacterium* strain carrying the virulence helper plasmid (Simon et al., 1983). Alternatively, the plasmid may be directly transformed into *Agrobacterium* by freeze-thaw transformation (Burow et al., 1990). The *Agrobacterium* is placed on suitably prepared plant material and the plasmid transforms individual cells which may grow out into transformed callus and/or plants.

The right border sequence from the *Agrobacterium* Ti plasmid and preferably the left one also are necessary for conjugation and plasmid integration into the plant genome; some adjacent sequences enhance or attenuate transfer (Zambryski et al., 1989). The borders are short direct repeats, 25 base pairs long. During bacteria-plant conjugation a site-specific nick occurs in the right sequence and the transferred DNA (T-DNA) leads off from this nick as a single strand with a 5' end, with transfer t

1.9.1 Plasmid pPCV701

The *Agrobacterium* plasmid pPCV701 (Figure 4) was developed by Koncz et al. (1987). Its features include: the *mas* bidirectional promoter, with adjacent unique restriction sites *Sal*I and *Bam*HI, polyadenylation sequences, kanamycin resistance and ampicillin resistance, and the Ti border sequences so placed that all these genes can be integrated into the plant genome. The plasmid size is about 9.8 kb.

For expression in a eukaryotic system, mRNAs require polyadenylate tails. cDNA clones lack the necessary signal sequences for polyadenylation. For transformation of cDNA genes into plants, therefore, polyadenylation sequences must be provided continguous to the insertion site. The polyadenylation sites of pPCV701 are derived from *A. tumefaciens* genes known to function in plants (Koncz et al., 1987).

Ampicillin resistance is derived from pBR322 of *E. coli*. The gene is integrated into the plant genome but not expressed there. It is used for selection of the plasmid during bacterial stages of construction.

Neomycin phosphotransferase (NPTII) is a kanamycin resistance marker derived from Tn5 of *E. coli* (Herrera-Estrella and Simpson, 1988). It is the most commonly used selective marker for plant transformation. The NPTII gene is under the control of the nopaline synthase promoter, from an *A. tumefaciens* opine gene highly expressed in plants.

Plasmid pPCV701 is used as part of a binary vector system with plasmid pMP90Rk, which carries trans-acting *vir* factors. These *vir* factors promote conjugation between the bacterial and plant cells.

1.9.2 The *mas* bidirectional promoter

The mannopine synthase (*mas*) promoter was taken from *A. tumefaciens*, where it promotes the synthesis of the opine mannopine. Thus it was known to function well and at high levels in plant cells. The fragment used was a bidirectional promoter, 479 base pairs in length (Velten and Schell, 1985). The P1 end of the bidirectional *mas* promoter

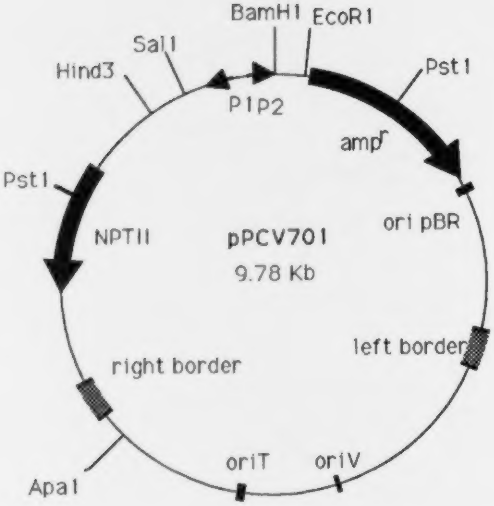

BamH1
EcoR1
Sal1
Hind3
Pst1
P1P2
amp^r
Pst1
ori pBR
NPTII
pPCV701
9.78 Kb
left border
right border
oriT
oriV
Apa1

Figure 4. *Agrobacterium* plasmid pPCV701.

has a polyadenylation sequence from the octapine synthase gene, while the P2 end has a polyadenylation sequence from yet another *Agrobacterium* gene functional in plant cells.

The expression pattern of the *mas* promoter in tobacco, controlling marker genes *lux*A and *lux*B, has been thoroughly studied. It is highly expressed in root tips, just outside vascular bundles, in the older parts of older leaves, in stigma and petal, but not pollen, ovary or seed (Langridge et al., 1989). The pattern is that expected of a promoter induced by auxin, and auxin indeed increases expression. Highest levels of expression are found in callus and suspension cells, which in tobacco are maintained on high levels of the auxin analogue, 2,4-D (Langridge et al., 1989). Cytokinins cause a smaller increase in expression. Langridge et al. (1989) report that the P2 promoter end acts at about the same intensity as P1 (Langridge et al., 1989), while Leung et al. (1991) report that the P2 promoter is 7-8 fold higher (Leung et al., 1991). Peach and Velten (1991), using 45 independently derived lines of tobacco callus carrying different marker genes at promoters P1 and P2, found that the ratio of activity of P1 to P2 varied from 0.05 to 49, indicating the magnitude of position effect. Leung et al. (1991) found expression patterns from promoters P1 and P2 to be similar in most tissues, except that P1 gave very little expression in root epidermis, primordia, and cap cells.

1.9.3 The luciferase gene from *Vibrio harveyi*

The luciferase gene (*lux*) encodes a light-emitting protein which is useful for screening for transformed material. *Lux*F results from the fusion of two genes, *lux*A and *lux*B, isolated from the marine bacterium *Vibrio harveyi* (Belas et al., 1982). The enzyme uses reduced FMN in the presence of long-chain fatty aldehydes (*n*-decanal is usually used) to emit photons in the 470-530 nm range. The reaction catalysed is:

$$FMNH_2+O_2+RCHO \dashrightarrow FMN+RCOOH+H_2O+0.1\ h\nu_{490nm}$$

(O'Kane et al., 1988).

*Lux*A and *lux*B produce proteins which form a 1:1 heterodimer (Koncz et al., 1987). Although the catalytic site is in the *lux*A portion, *lux*B is nonetheless required for luminescence (Koncz et al., 1987; Olsson et al., 1988). A. Escher constructed a fusion gene (2.2 kb) and protein, *lux*F (correctly, *fab*2, but commonly called *lux*F) (78 kD) from these two, which functions equally well at 24^{o}C, though the activity drops to near zero at 37^{o}C (Escher et al., 1989). The addition of excess luxB protein promotes luminescence at 37^{o}C, suggesting that *lux*B may be required for correct folding of the catalytic region (Escher et al., 1989).

Activity of the *lux* gene is detected by light emission in the presence of *n*-decanal and reduced FMN. *N*-decanal is somewhat toxic, but is volatile and readily crosses cell membranes and penetrates tissues. The light emitted is occasionally visible to the eye (Legocki et al., 1986), but is normally examined using photon detectors or on film (Legocki et al., 1986; Langridge et al., 1992). Most frequently used is a luminometer which is a photon-counting chamber. One light unit (LU) represents 1.6×10^6 photons/sec (Langridge et al., 1989); transformed tobacco may produce 4 to 26 LU/mg fresh weight (Koncz et al., 1987).

Because of its flexibility and simplicity of detection, the luciferase system is increasingly used as a reporter gene in plants and other systems. It lacks the resolution at the cellular level of β-glucuronidase (GUS), but can be assayed in living tissue which can survive the assay (Langridge et al., 1991), unlike almost any other reporter system currently available.

2. Materials and Methods

2.1 Growth of crop seedlings with weed extracts and allelochemicals

Almost all the work involving crop seedlings employed the same general experimental methods. The general method is described in detail; slight variants are noted where appropriate and listed in Table 4. Seed sources are listed in Table 2.

About 6 cm of medium grade vermiculite was placed in a standard 15 cm test tube, capped or covered with aluminum foil, and autoclaved for 15 min at 121°C. Phenolic compounds were dissolved in 1X or 0.5X Hoagland's solution (Wetter and Constabel, 1982) and autoclaved for 15-20 min at 121°C. Levels of phenolic compounds which produced a broad range of effects were chosen following preliminary experiments (not shown).

Weeds were collected from local fields. Weed extracts were prepared by pushing fresh weed tops (top down) or roots into distilled water, avoiding exposure of cut surfaces to water as much as possible, at 4-10% fresh weight per volume. After overnight or longer soaking at 4°C or 21°C, the extract was poured off, filter-sterilized or autoclaved in some cases, and refrigerated or frozen until used. This method was intended to maximize resemblance to the natural situations of rainwater or groundwater extraction of live weeds or fresh residues. Some wild oat samples were extracted in boiling water for 2 hours. The weed extracts were mixed with 10X Hoagland's solution 1:19 or 1:9 to give 0.5X or 1X Hoagland's solution with 0.9X or 0.95X weed extract.

One seed of the species being studied was then placed on the vermiculite in each labelled tube and 5 ml of sterile nutrient solution added. This amount saturated the vermiculite without leaving excess liquid. After early experiments established that contamination did not affect live seedlings, seeds were not sterilized.

Tubes were held upright in racks, in every second row only, to maximize and even out light penetration as much as possible. Tubes were placed in a randomized complete block design, but block layout was not recorded at measuring.

In most experiments tubes were then capped or covered with aluminum foil and kept in a dark cupboard until germination began. They were then moved to a growth chamber with a 16/8 hour light/dark cycle, 19-26°C/19°C temperature cycle, and about 22,000 lumens/m^2, where they were grown until measured. Usually, aluminum foil or caps were removed at this time, sometimes a day later. Flax generally was kept 4 days in the dark; grains, 3 days.

Flax seedlings were measured at 14-15 days; grains, at 10 days. Shoot length of each germinated seedling was measured. The seedlings were then gently removed from the tubes, the vermiculite shaken and picked away and roots disentangled, extended, and measured. The roots were then severed. Roots and shoots were placed on aluminum foil trays to be dried at 55-60°C overnight. Collective dry weight and number were recorded. The average, standard deviation, standard error and percentage of control were found for every eligible measurement.

In all measures where the average height was substantially more than 20 mm, seedlings less than 20 mm high were excluded from the data, on the basis that these must have sprouted from defective, late-germinating seeds. This was done only where there was no doubt that normal seedlings were at least 2-3 times taller; where doubt existed, they were included. This was uncommon with flax which germinated very uniformly, but was moderately common with grains. Other reasons for exclusion were that the seedling died (very rare) or two seedlings were in a tube (about 1% of tubes).

The pH of the phenolic solutions was recorded. Either the pH was adjusted to about 5.8 or a pH control at the lowest pH recorded was performed. After it became evident that starting pH was of little significance, these pH controls were sometimes omitted.

Table 2. Plant varieties.

Material	Variety	Source
Avena fatua		gift of Dr. J. O'Donovan
Avena sativa	Cascade	gift of Alberta Agriculture
	Dumont	"
Cirsium arvense		gift of D. Trudzik
Hordeum vulgare	Conquest	gift of Alberta Agriculture
	Elrose	"
	Galt	"
	Johnston	"
	Leduc	"
Linum halogynum		Dr. W.E. Smith collection
Linum pallescens		"
Linum usitatissimum	Mcgregor	purchase
	Raja	Dr. W.E. Smith collection
	Abyssinian Brown	"
	Indian Type 8	"
Nicotiana tabacum	SR1	gift of Dr. A. Szalay
	#5 (SR1/*lux*A/*lux*B NPTII)	"
Triticum aestivum	Katepwa	gift of Henkel Seed Farm

Table 3. Bacterial strains and plasmids.

Material	Name/Genotype	Source or Reference
Bacterial strains		
A. tumefaciens	GV3101 pMP90Rk	gift of Dr. A.A. Szalay
E. coli	SM10	"
	DH5α	Sambrook et al, 1989
	HB101	"
Plasmids		
pCCP1	*Cyp*1A1 in *Eco*R1/*Bam*H1 cassette	gift of Dr. D. Pompon
pCDP450	*Cyp*2A5 in *Eco*R1/*Eco*R1 cassette	gift of Dr. M. Negishi
pPCV701	*A. tumefaciens* vector	gift of Dr. A.A. Szalay
pPCV701*lux*A*lux*B	"	"
MpUC19	modified pUC19	gift of P. Manivasakam
p23	intermediate construct	this thesis
p25	"	"
p32	"	"
p2322	"	"
p2319	"	"
p4	pPCV701*Cyp*1A1P2 forward*lux*FP1 forward	this thesis
p421	isolate of p4	"
p471	"	"
p481	"	"
p14	pPCV701*Cyp*1A1P2 reverse*lux*FP1 forward	this thesis
p143	isolate of p14	"
p146	"	"
p10	pPCV701*Cyp*2A5P2 forward*lux*FP1 forward	this thesis
p105	isolate of p10	"
p106	"	"
p107	"	"

Table 4. Experimental conditions for phenolic effects on seedlings.

Species	Expt. #	pH	Seedlings/sample shoot	root	Hoagland's concentration	Time, days
Flax McGregor	119	unadjusted	20	8	0.5	14
	120	5.7	20	8	0.5	14
	51	unadjusted	12	12	1	15
	82	unadjusted	12	8	0.5	14
	122	unadjusted	20	8	0.5	14
	220	6.5	20	20	0.5	13
	103	5.8	20-40	8	0.5	14
	24	unadjusted	6 - 12	0	1	13
	30	unadjusted	6-12	0	1	14
Wheat Katepwa	102	5.8	20	8	0.5	10
	116	unadjusted	20	8	0.5	10
	101	unadjusted	20	8	0.5	10
	54	unadjusted	12	n.d.	1	10
	220	6.5	20	20	0.5	10
	103	5.8	20-40	8	0.5	10
	25	unadjusted	6-12	n.d.	1	10
	30	unadjusted	6-12	n.d.	1	10
Oats Cascade, Dumont	97	5.2	20	8	0.5	15
Barley Leduc	220	6.5	20	20	0.5	10

Leather and Einhellig (1986) suggest that osmotic potential should also be controlled. However, at the concentrations used it appeared not to be a factor, and therefore osmotic potential was not controlled. Of course, phenolic compounds in the field will contribute to both soil pH and osmotic potential.

The number of seedlings per treatment varied from 12 to 20 and is noted in Table 4.

Tobacco was grown in 35x100 mm test tubes. Fifteen ml of 1X Hoagland's solution with or without ferulic acid in about 3 cm of vermiculite was used. Non-sterile seeds were placed singly in tubes, which were randomized and grown as previously described. Shoot measurements only were made.

Tobacco germination was tested on agar plates rather than in vermiculite, since germination is more uniform on agar. Germination was assayed in standard Petri dishes containing about 22 ml of 0.5X MS salts (without sugar, hormones, antibiotics or vitamins) plus phenolic compounds and 0.8% agar. Phenolic compounds (*p*-coumaric, ferulic and vanillic acids at 1 and 4 mM and coumarin at 0.1 and 1 mM) were added prior to autoclaving. pH was not adjusted, but a control at pH 3.1 was performed. Non-sterile seeds were placed on the plate surfaces, 10 seeds per plate, 3-5 plates per treatment. Seeds were grown at 23°C under continuous room light. Numbers of germinated seeds were scored at 4, 6, 9, and 13 days.

Examination of intraspecific variation was conducted very similarly to earlier flax trials. All the flax varieties except McGregor were from the University of Alberta flax collection of the late Dr. W.E. Smith. Barley seed was donated by Alberta Agriculture. Fifty flax or 20 barley seeds per treatment per variety were placed singly in test tubes containing vermiculite and Hoagland's solution with or without phenolic compounds. Flax was treated with 1 mM *p*-coumaric acid adjusted to pH 5.9. Barley was treated with 0.95X wild oat shoot extract, 0.5 mM scopoletin or 2 mM *p*-coumaric acid adjusted to pH 5.8. Moderately harsh treatments were used so that differences would be maximal. The test tubes were randomized and placed in every second row of test tubes racks which were placed in the dark at room temperature. When germination began, all the tubes were moved to the growth chamber until measured. Shoot height of all the seedlings and root length, shoot and root dry weight of a randomly selected sample were measured. Flax was measured at 21 days and later; barley, at 10 days.

In the case of flax, 20 randomly selected seedlings from each combination of treatment with variety were then potted, 5 per randomly placed pot, and grown to maturity in the growth chamber. The seedlings previously treated with *p*-coumaric acid continued to have 10 ml of 10 mM *p*-coumaric acid added at weekly intervals, to a final total of 0.05 g/plant. Shoot height was measured periodically and height, stem number and seed weight measured at harvest at 19 weeks.

Statistical analysis was carried out by analysis of variance (ANOVA) and by Newman-Keuls test for ranked differences (Zar, 1984).

2.2 Cloning of P450 genes

2.2.1 Outline of plasmid construction and transformation

The steps of plasmid construction and plant transformation were as follows (Figure 5):

1. Plasmids carrying the P450 genes were restricted with *Bam*HI and *Eco*RI (*Cyp*1A1) or *Eco*RI alone (*Cyp*2A5), releasing the P450 genes. Fragments were separated by agarose gel electrophoresis and the P450 fragments recovered from the gel.
2. Plasmid pPCV701 restricted at the unique *Bam*HI site adjacent to P2.
3. *Cyp*1A1 was made blunt ended by Klenow fragment, ligated to *Bam*HI linker fragment,

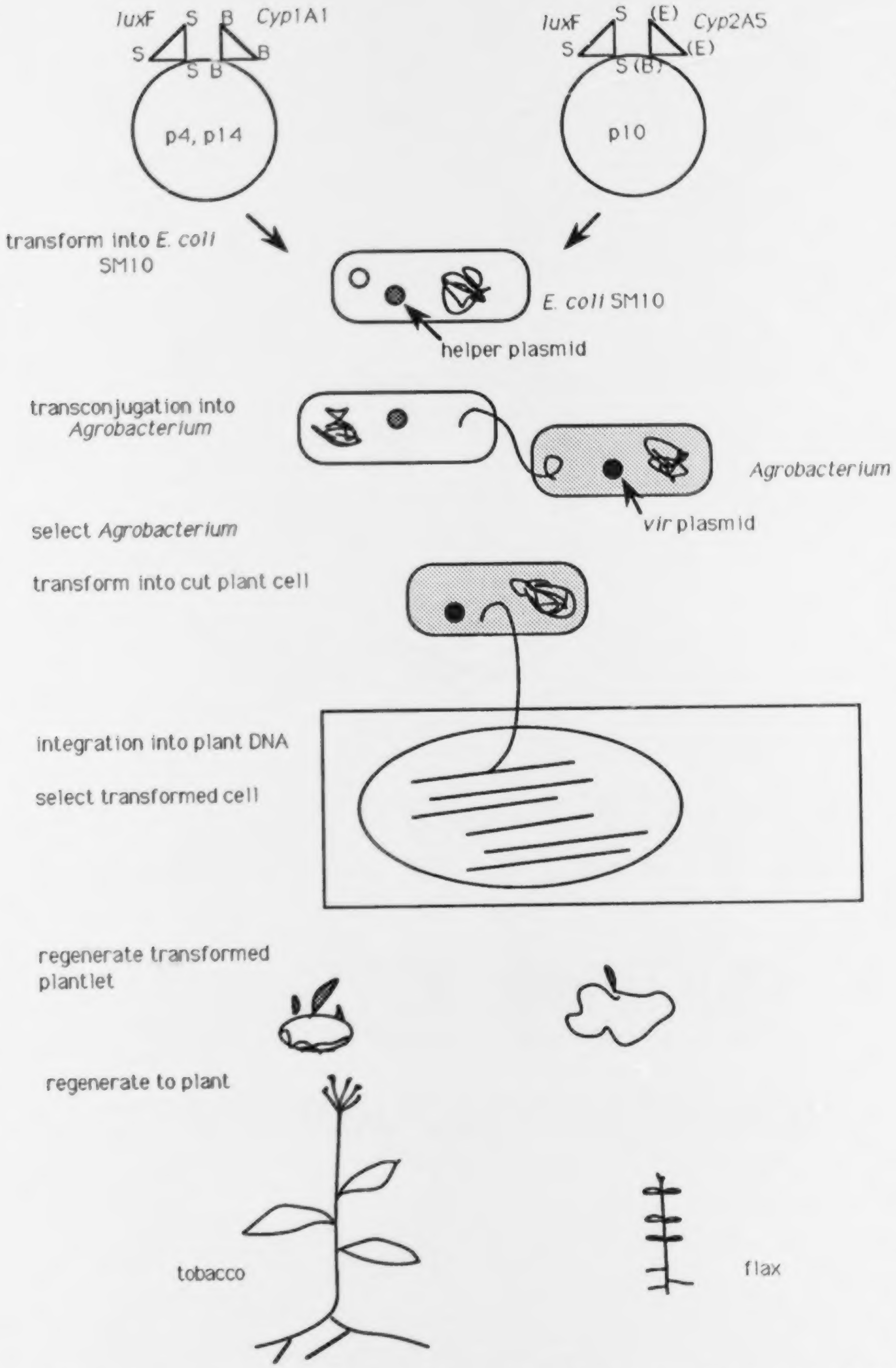

luxF
S
S
B
Cyp1A1
S
B
B
p4, p14
luxF
S
S
(E)
Cyp2A5
S (B)
(E)
p10
transform into E. coli SM10
E. coli SM10
helper plasmid
transconjugation into Agrobacterium
Agrobacterium
vir plasmid
select Agrobacterium
transform into cut plant cell
integration into plant DNA
select transformed cell
regenerate transformed plantlet
regenerate to plant
tobacco
flax

Figure 5. Outline of P450 gene cloning and transformation into plants.

digested with *Bam*HI and then ligated to calf-intestinal-phosphatase (CIP)-treated derivative of pUC19 and transformed into *E. coli* DH5α, selecting for ampicillin resistance and white colour. DNA was isolated and the fragment digested out of this vector with *Bam*HI, ligated to CIP-treated pPCV701 and transformed into DH5α, selecting for ampicillin resistance.

4. *Cyp*2A5 and pPCV701 were made blunt ended by Klenow fragment, 701 was treated with CIP and the fragments were then ligated and transformed into *E. coli* HB101 with selection for ampicillin resistance.
5. The new constructs were examined by restriction analysis to determine gene orientation, then restricted with *Sal*I to open the unique *Sal*I site at P1, and the linearized plasmid dephosphorylated.
6. The *lux*F fragment was cut out of an *Agrobacterium* plasmid carrying *lux*F at the *Sal*I site, the fragments were separated by agarose gel electrophoresis and the *lux*F fragment was recovered from the gel.
7. The *Agrobacterium* plasmids carrying P450 genes were opened with *Sal*I and ligated to the *lux*F fragment, then transformed into HB101, with selection for ampicillin resistance.
8. These constructs were examined by restriction analysis to check presence and orientation of the genes.
9. For the *Cyp*1A1 construct, the plasmids were used to transform the *E. coli* strain SM10, which carries genes promoting promiscuous transconjugation of other resident plasmids, using selection for ampicillin resistance (Simon et al., 1983). Transformed SM10 was mixed with *A. tumefaciens* strain GV3101, which carries the kanamycin-resistant *vir* plasmid pMP90Rk, and allowed to conjugate. Transconjugants were double-selected by kanamycin and carbenicillin, which is more effective in *Agrobacterium* than is ampicillin.
10. For the *Cyp*2A5 construct, the plasmid was directly transformed into *A. tumefaciens* GV3101 by freeze-thaw transformation (Burow et al., 1990) or by transconjugation.
11. Transconjugants or transformants were assayed for light emission in the presence of *n*-decanol, under a low-light imager.
12. Luminescent *A. tumefaciens* were used to transform plants. For tobacco, leaf discs of sterile tobacco were cut and mixed with *A. tumefaciens*, grown for three days on Murishige-Skoog (MS) (Wetter and Constabel, 1982) plates in the absence of selection, then transferred to MS containing kanamycin to select against nontransformed material, cefuroxime to select against bacteria, and appropriate phytohormones to promote leaflet formation. Tobacco kanamycin-resistant leaflets were grown to a suitable size and transferred to rooting medium and when rooted to soil.
13. For flax, 2-day-old sterile hypocotyls were cut into 1-2 mm bits and mixed with *Agrobacterium* and treated similarly to tobacco. Flax kanamycin-resistant leaflets were multiplied in culture, then transferred to rooting medium.

2.2.2 Construction of plasmids p4 and p14 (pPCV701*Cyp*1A1$p_2$luxFp_1)

Most cloning procedures used standard methods such as those described in Maniatis et al. (1982), Dillon et al. (1985), and Sambrook et al. (1989), or minor variations thereof. Ligations followed the recommendations of these authors and of Revie et al. (1988). DNA was quantitated or its quality examined by agarose gel electrophoresis using 0.7% agarose gels in Tris-acetate buffer (TAE), with DNA fragment markers. Restriction and other enzymes were obtained from Gibco BRL, Boeringher Mannheim and Pharmacia. Enzymes were used according to manufacturer's suggestions in most cases. Chemicals and materials were from various manufacturers, chiefly Sigma, Fisher, Boeringher Mannheim, New England Nuclear (NEN) and Gibco

BRL.

The object of this construction was to place *Cyp*1A1 and *lux*F (as a marker gene) under the control of the bidirectional *mas* promoter as shown in Figure 6. *Cyp*1A1 was placed under the promoter P2 both in correct and reverse orientation. The construct with reverse orientation could serve as a negative control for later assays. Plasmid pCCP1, carrying the P450 gene as a *Bam*HI-*Eco*RI fragment, was the gift of Dr. D. Pompon and is described in Cullin and Pompon (1988). This plasmid was designed for expression of the P450 gene. The construct appeared suitable for expression in plants.

The presence of an *Eco*RI restriction site in the *lux*F fragment, and the absence of *Sal*I sites in the P450 gene, dictated that the *Bam*HI-*Eco*RI fragment first be placed in the unique *Bam*HI site of pPCV701 under *mas*P2, in correct and reverse orientations. This was accomplished using a *Bam*HI linker. The *lux*F *Sal*I-*Sal*I fragment was then placed in the unique *Sal*I site of pPCV701 under *mas*P1. *Lux*F was only of interest in the correct orientation.

2.2.2.1 Insertion of *Cyp*1A1 into pPCV701

Plasmid pCCP1 was digested with *Eco*RI and *Bam*HI and the digestion products were separated on a preparative agarose gel. The 1.86 kb fragment of interest was sliced out of the gel and extracted from the agarose using Gene-Clean 2 (Bio 101).

The *Bam*HI linker was prepared by phosphorylation of its ends with T4 polynucleotide kinase and ATP. The ends of the 1.86 kb fragment were filled in using Klenow fragment of *E. coli* DNA polymerase I according to Maniatis et al. (1982), but using the Klenow buffer of Dillon et al. (1985). The now blunt-ended fragment was ligated to the *Bam*HI linker at a linker:insert ratio of 1:2 by weight, giving a large excess of *Bam*HI linker ends, as suggested by Dillon et al. (1985). After ligation overnight at 14°C, the DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) followed by ethanol-salt precipitation (PCE) (Maniatis, 1982). The DNA was then digested with an excess of *Bam*HI to remove the excess linker fragments and generate *Bam*HI termini. The 1.86 kb fragment was isolated by preparative gel electrophoresis followed by extraction with Gene-Clean 2.

Meanwhile the vector was prepared by digestion to completion with *Bam*HI. The vector, MpUC19, used was a pUC19 modified by removal of part of the polylinker site from *Hin*dIII to *Xba*1, but retaining the *Bam*HI site (P. Manivasakam, personal communication). The DNA was dephosphorylated using CIP according to Maniatis et al. (1982). The enzyme was removed by PCE treatment, so that no dephosphorylation of the insert would occur.

Vector and insert were mixed in 1:1 or 1:3 molar ratios and ligated overnight at 14°C, with suitable controls. A portion of each ligation product mixture was used to transform competent DH5α. Bacteria were plated on LB with 50 µg/ml ampicillin, 62 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) and 25 ng/ml isopropyl-thiogalactoside (IPTG), freshly prepared.

Religated vectors in DH5α were expected to produce pale blue colonies; vectors with insert, white colonies. All colonies recovered from the vector-insert mixtures were white, indicating that an insert was present.

The apparent recombinants were restreaked and minipreps of plasmid DNA made by the procedure of Morelle (1989), which yields enough good quality DNA for several restriction digests with a minimum number of steps. DNA was isolated from liquid overnight cultures or 2-day-old streaks resuspended in lysis buffer.

Putative recombinant plasmids were digested with *Bam*HI and sorted by electrophoresis. The following bands were expected and found:

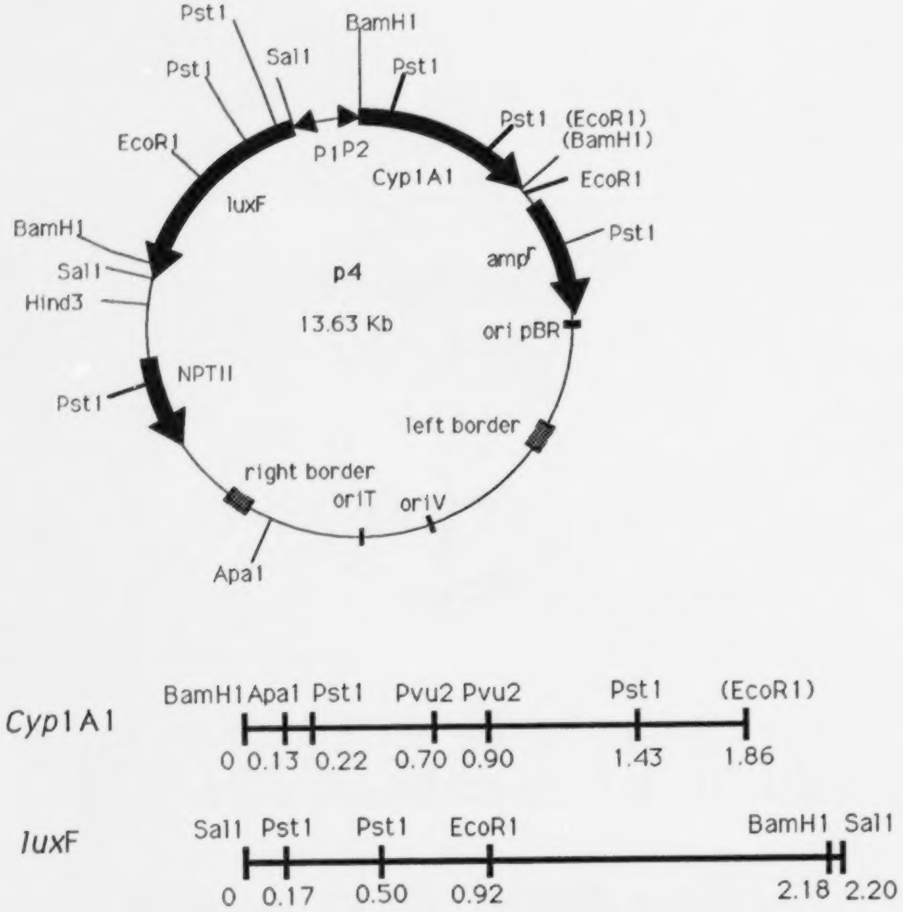

Pst1
BamH1
Pst1
Sal1
Pst1
Pst1 (EcoR1)
(BamH1)
EcoR1
EcoR1
P1P2
Cyp1A1
luxF
Pst1
BamH1
Sal1
Hind3
p4
13.63 Kb
ampr
ori pBR
NPTII
Pst1
left border
right border
oriT
oriV
Apa1
Cyp1A1
BamH1 Apa1 Pst1 Pvu2 Pvu2 Pst1 (EcoR1)
0 0.13 0.22 0.70 0.90 1.43 1.86
luxF
Sal1 Pst1 Pst1 EcoR1 BamH1 Sal1
0 0.17 0.50 0.92 2.18 2.20

Figure 6. *Agrobacterium* plasmid p4, with *Cyp*1A1 and *lux*F controlled by the bidirectional *mas* promoter. p14 is identical except that *Cyp*1A1 is inserted in the opposite orientation. Sites indicated in brackets were eliminated during construction.

		MpUC19		recombinant p32			
*Bam*HI	expected	2.7kb		1.86	2.7		
	observed	2.6		1.7	2.6		
*Pst*I	expected	uncut		1.21	3.35		
	observed	uncut		1.2	3.2		
*Pvu*II	expected	2.38	.32	2.38	1.1	.88	.20
	observed	2.2	.4	2.2	.1.0	.8	.3
Total	expected	2.7		4.6			
	observed	2.6		4.5			

Plamid p32 showed the expected fragment sizes and was used for the next stage of construction: transfer of the 1.86 kb *Cyp*1A1 fragment, with *Bam*HI sites at both ends, to the *Agrobacterium* vector pPCV701.

Plasmid p32 DNA was digested to completion with *Bam*HI and the 1.86 kb fragment extracted from a preparative gel by Gene-Clean 2. Vector pPCV701 was digested to completion with *Bam*HI, the vector dephosphorylated and the DNA extracted by PCE. Vector and insert were combined in 1:1 and 3:1 molar ratios and ligated with several controls. One fifth of each ligation mixture was transformed into competent DH5α and the transformation mixtures split into 0.1 and 0.9 ml aliquots and plated on LB plates with 100 μg/ml ampicillin. Few transformants were recovered owing to low transformation and ligation efficiency. pPCV701 typically transforms at about 10% of the efficiency of such standard *E. coli* vectors as pUC19.

Twenty-four putative transformants were restreaked and miniprep plasmid DNA prepared and digested with *Bam*HI. Eighteen of these 24 contained the 1.86 kb P450 fragment. DNA preparations were digested with *Eco*RI; 6 of 12 transformants proved to have the insert in the correct orientation and 6 of 12 in the reverse orientation. Fragment sizes were as follows:

		pPCV701	p23 correct orientation		p25 reverse orientation	
*Bam*HI	expected	9.8 kb	9.8	1.9	9.8	1.9
	observed	9.8	9.8	1.8	9.8	1.8
*Eco*RI	expected	9.8	11.6	.2	9.8	1.9
	observed	9.8	>10	(off gel)	9.7	1.9

The presence of the P450 gene in these constructs was confirmed by DNA dot blots and Southern blots of *Apa*I digests. The probe used was the 1.86 kb *Cyp*1A1 fragment, oligolabelled with α^{32}P dCTP using a BRL kit according to manufacturer's instructions. The gel was blotted onto Gene-Screen Plus (NEN) and hybridized overnight at 42^{o}C as suggested by the manufacturers. Dot-blots were prepared on Gene-Screen Plus and similarly probed.

2.2.2.2 Insertion of *lux*F

The constructs p23 (*Cyp*1A1 in correct orientation) and p25 (*Cyp*1A1 in reverse orientation) were then used as vectors into which the insert *lux*F was inserted. Only *lux*F in the correct orientation was of interest. Plasmid DNA from minipreps was used. It was digested to completion with *Sal*I, dephosphorylated, and extracted by PCE.

The *lux*F fragment was isolated from plasmid p701*lux*FP1 by digestion to completion with *Sal*I. The fragments were separated on a TAE preparative gel and the correct fragment (2.2 kb) sliced out and extracted using Gene-Clean 2.

The *lux*F fragment and *Sal*I-cut p23 or p25 were mixed in 1:1, 3:1 and 10:1 molar ratios and ligated as before. One fifth of the ligation products were transformed into competent HB101or DH5α. The transformation mixtures were apportioned as previously and plated on LB with 100 µg/ml ampicillin. Transformants were recovered.

Dephosphorylation proved to be about 90% complete. Since the first transformants tested did not show digestion patterns indicating insertion, colony lifts onto Gene-Screen Plus were performed according to Dillon et al. (1985). The membranes were probed with the *lux*F fragment oligolabelled with a BRL kit. Hybridization followed Gene-Screen Plus instructions. 10-20% of the transformants hybridized. The originals of these colonies were restreaked, plasmid DNA prepared, and DNA digested with *Bam*HI. Digestion products with *lux*F in the correct orientation were as follows:

		p421, p471, p481 (from p23)	p143, p146 (from p25)
*Bam*HI	expected	9.9 2.6 1.9 kb	9.9 2.6 1.9
	observed	10 2.6 1.9	10 2.6 1.9

Plasmids p421, p471 and p481 (equivalent isolates of p4) carrying *Cyp*1A1 and *lux*F in the correct orientation and p143 and p146 (equivalent isolates of p14) with *Cyp*1A1 in the reverse orientation and *lux*F in the correct orientation, were then transformed into *E. coli* SM10 (Simon et al., 1983). This strain carries conjugative functions which act in *trans* and kanamycin resistance. Recovery from transformation was for 75 minutes at 30°C. Transformants were selected on LB medium with 100 µg/ml ampicillin and 25 µg/ml kanamycin.

Ampicillin-resistant SM10 derivatives were mixed with *A. tumefaciens* GV3101 carrying the virulence plasmid pMP90Rk, the mixture dropped onto LB and grown 2 days at 29°C. Toothpickfuls of bacteria were diluted 10^{-2} and 10^{-4} and plated on a medium selective for transformed *Agrobacteria*, LB with 50 µg/ml rifampicin, 100 µg/ml carbenicillin and 25 µg/ml kanamycin. Transconjugation rates appeared low; about 10 colonies from each plasmid were obtained. These were restreaked and one-day-old replica streaks examined with a low light imager (Langridge et al., 1991). All isolates emitted light. A few of the brightest were used for transformation of tobacco and flax.

2.2.3 Construction of plasmid p10 (p701*Cyp*2A5$p_2$luxFp_1)

The object of this construction was nearly identical to that of p4: to place *Cyp*2A5 and *lux*F under the control of the bidirectional *mas* promoter, as shown in Figure 7.

Plasmid pCDP450 was donated by Dr. M. Negishi. *Cyp*2A5 was cloned as cDNA (Squires and Negishi, 1988) and the published sequence contains three *Eco*RI restriction sites. These were removed by site-specific mutation without altering the encoded protein sequence (M. Negishi, personal communication) and the cDNA placed as an *Eco*RI-*Eco*RI cassette in plasmid pCDP450.

As with the previous construct, it was most suitable to place the *Eco*RI-*Eco*RI *Cyp*2A5 fragment in the *Bam*HI site of plasmid pPCV701 first, using blunt-end ligation, then to place the *Sal*I-*Sal*I *lux*F fragment in the *Sal*I site.

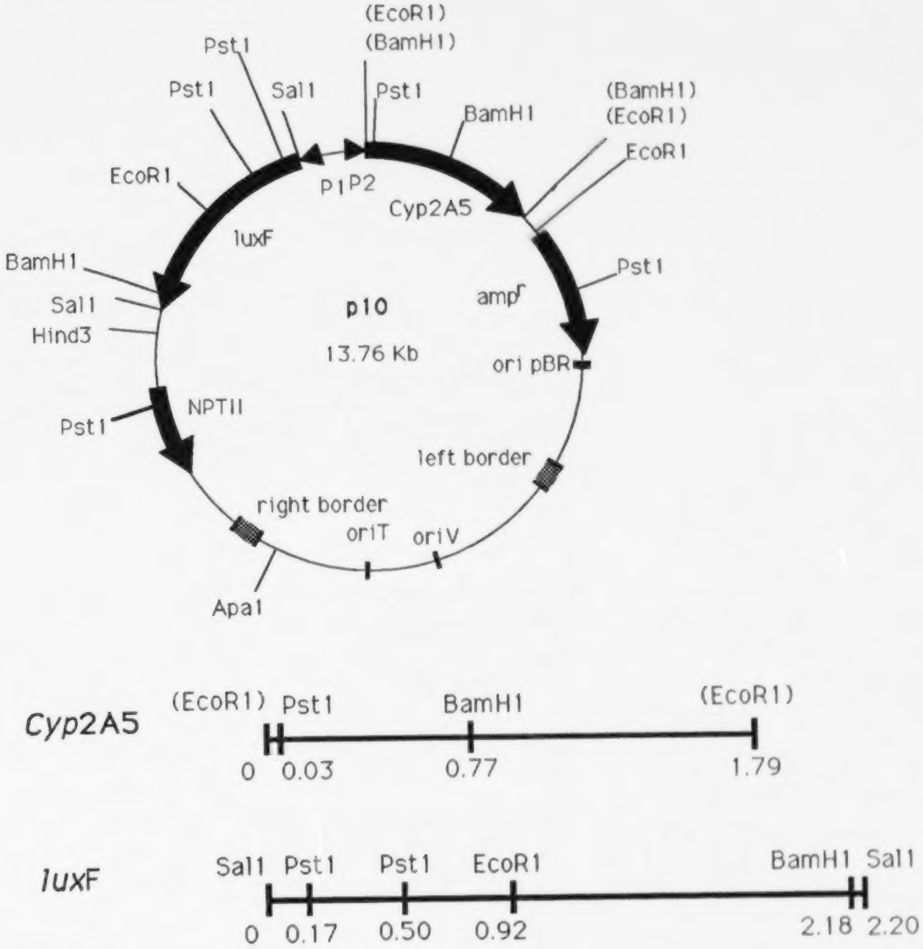

(EcoR1)
(BamH1)
Pst1
Pst1
Sal1
Pst1
BamH1
(BamH1)
(EcoR1)
EcoR1
EcoR1
P1
P2
Cyp2A5
luxF
BamH1
Sal1
Hind3
Pst1
amp^r
p10
13.76 Kb
ori pBR
Pst1
NPTII
left border
right border
oriT
oriV
Apa1
Cyp2A5
(EcoR1)
Pst1
BamH1
(EcoR1)
0
0.03
0.77
1.79
luxF
Sal1
Pst1
Pst1
EcoR1
BamH1
Sal1
0
0.17
0.50
0.92
2.18
2.20

Figure 7. *Agrobacterium* plasmid p10 with *Cyp*2A5 and *lux*F controlled by the bidirectional *mas* promoter. Sites indicated in brackets were eliminated during construction.

2.2.3.1 Insertion of *Cyp2A5*

This construct was made using almost the same methods as the previous construct. Its construction will therefore be described more briefly. The chief differences are that the *Cyp*2A5 construct was made using blunt end ligation, without *Bam*HI linker, and the final plasmid was transformed into *Agrobacterium* by a freeze-thaw method (Burow et al., 1990) rather than by SM10 transconjugation.

Plasmid pCDP450, carrying *Cyp*2A5, was digested to completion with *Eco*RI and the fragments separated by preparative gel electrophoresis, the 1.79 kb fragment of interest sliced out of the gel and extracted from the agarose with Gene-Clean 2. The 1.79 kb fragment was then made blunt-ended by filling in with Klenow fragment. The reaction was ended by brief heating at 75°C followed by PCE.

Plasmid pPCV701 was digested to completion with *Bam*HI, similarly filled in, dephosphorylated with CIP, cleaned with PCE, and ligated to the insert fragment as described above. The DNA concentration was increased as much as possible, to maximize the possibility of ligation between the blunt ends. Insert:vector molar ratios of 1:1, 3:1 and 10:1 were used.

The ligation products were transformed into HB101 as before and numerous transformants recovered. Of these, the first 16 plasmids examined lacked the insert, presumably because of the incomplete (90%) CIP treatment allowing religation of the vector. I therefore performed colony lifts onto Gene-Screen Plus; these were probed with ^{32}P-labelled *Cyp*2A5 fragment. About 5% of colonies hybridized to the probe. Plasmid minipreps of these were prepared and digested with *Sal*I and *Eco*RI to find which ones had insertions. They were then digested with *Pst*I or *Pst*I and *Eco*RI to test the orientation of insertion.

Expected and observed digestion products for *Cyp*2A5 insertion in correct and reverse orientations were compared.

		pPCV701	p2322 correct orientation	p2319 reverse orientation
*Sal*I and *Eco*RI	expected	9.0 0.8 kb	9.0 2.6	9.0 2.6
	observed	9 0.8	9 2.6	9 2.6
*Pst*I	expected	6.8 2.7	6.8 2.7 1.8	6.8 3.5 1.0
	observed	7.0 2.8	7.0 2.1 1.7 0.8	7.0 2.3 1.1 0.8
*Pst*I and *Eco*RI	expected	6.8 2.1 .6	6.8 2.1 1.8 .6	6.8 3.5 .6 .4
	observed	7.0 1.8 0.8	7.0 1.6 1.3 0.8	7.0 2.4 0.9
Total	expected	9.8	11.6	11.6
	observed	10.0	13	13

Most fragments were as expected. However, a *Pst*I site not occurring in the published sequence has appeared. Sequencing showed no change in the amino acid sequence at this point.

2.2.3.2 Insertion of *luxF*

The *lux*F gene was then inserted into plasmids p2319 (*Cyp*2A5 in correct orientation) and p2322 (*Cyp*2A5 in reverse orientation), in the correct orientation only. The *lux*F fragment was that previously prepared. The plasmids were digested to completion with *Sal*I, plasmids cleaned with PCE, dephosphorylated with CIP, the reaction terminated as suggested by Maniatis (1982), and cleaned again with PCE. Ligation was like those previously described. The ligation mixtures were transformed into competent HB101 and selected on LB with 100 µg/ml ampicillin.

Plasmid DNA from 31 transformants was prepared and digested with *Bam*HI, yielding the following products:

		p105, p106, p107 (from p2322)	(from p2319)
*Bam*HI	expected	9.1 2.7 kb	9.1 2.7
	observed	8.9 3.2	none found

Six of 13 minipreps from p2322 constructs carried *lux*F fragments in the correct orientation, but 0 of 60 minipreps from p2319 appeared to have *lux*F. Rather than repeat the construction I used the previous constructs p143 and p146, carrying *Cyp*1A1 in reverse orientation, as controls for the successful *Cyp*2A5 constructs p105, p106 and p107.

Plasmids of the p10 group, p105, p106 and p107, were conveyed into *Agrobacterium* by freeze-thaw transformation (Burow et al., 1990) rather than by transconjugation from SM10. This method is claimed to produce more virulent *Agrobacterium* as well as to minimize the number of steps, although it is acknowledged to be very inefficient (Burow et al., 1990). Log phase cells were washed, resuspended in cold LB medium, mixed with 0.5-1 µg DNA, held on ice 5-15 min., frozen in liquid nitrogen 5-10 minutes, heat shocked 5 minutes at 37°C, then incubated 5 hours at 28°C before plating on LB medium with 50 µg/ml rifampicin, 25 µg/ml kanamycin, 25 µg/ml gentamycin and 100 µg/ml carbenicillin, at 28°C. About 56 *Agrobacterium* transformants were recovered from 3 µg of DNA. Lower rates of transformation with p701*lux*FP1 and pPCV701 were obtained. A background lawn of escapes appeared two or three days after the transformants. Transformants were restreaked twice and the best growers tested after overnight growth for luciferase activity with a low-light imager. Eight of 10 *Cyp*2A5 constructs tested shone; the brightest were used for plant transformation.

I should note that I have not since had much success with transformation by the freeze-thaw method, nor have several other people who have tried it. Since it succeeded in this instance, albeit at very low frequency, it must need optimization of some unknown critical factors.

2.3 Plant transformation

Luminescent *A. tumefaciens* carrying the three constructs p4, p14 and p10 and some control constructs such as pPCV701*lux*F$_{P1}$ were used to transform tobacco and flax.

Tobacco was transformed using the leaf-disc transformation method of Horsch et al. (1985). Sections from young sterile tobacco leaves were excised, held in a 1:1 dilution of overnight-grown *Agrobacteria* for 3-5 minutes at 24°C, blotted gently, and placed on nonselective plates (MS (Wetter and Constabel, 1982) with 1 µg/ml 6-benzylaminopurine (BAP), 0.1 µg/ml β-naphthyleneacetic acid (NAA)) in the dark for 3 days at 24°C to allow bacterial proliferation, then transferred to selective plates containing, in addition to the above, 100 µg/ml kanamycin to kill nontransformed plant material and 400 µg/ml cephoroxim to prohibit growth of free-living *Agrobacteria*. Leaf sections were thereafter maintained in moderate light at 24°C. Leaf sections formed small amounts of callus along cut edges, followed by formation of leaflets. These were excised at 5-10 mm and transferred individually to fresh medium in 35x100 mm test tubes. They grew rapidly and, when large enough (20-30 mm), were transferred to hormone-free rooting medium containing kanamycin. Root formation in the presence of kanamycin is slower but a good indication of transformation. Over half of the excised shoots rooted successfully and a number were washed free of agar and transferred to vermiculite wetted with 0.5X Hoagland's solution (Wetter and Constabel, 1982) in 800 ml plastic beakers, covered with Saran wrap. When well established the plants were transplanted to light soil:sand:vermiculite mixture of varying composition. They were at first protected from dessication by plastic coverings which were gradually withdrawn. At each stage the success rate was high. All plants matured and set seed. Self-fertilization was enforced by enclosing unopened buds in wax paper envelopes. Seed production was reduced under these circumstances but was still substantial. All mature seed germinated at a high rate.

Flax transformation and regeneration is not as simple as that of tobacco. I attempted to transform flax by the methods of Jordan and McHughen (1988a) and Draper et al. (1988). Jordan and McHughen's method of epidermal peeling produced a high death rate and a good deal of callus among the survivors, and a small number of nontransformed shoots (escapes). It appeared that this method was too harsh and therefore unlikely to be successful.

The method of Draper et al. (1988) was performed much as described. Flax seed was sterilized in ethanol 1-2 minutes followed by 20-25 minutes in 25% commercial bleach, rinsed thoroughly with sterile distilled water, then placed on sterile vermiculite wetted with sterile water or nutrient solution. At 3 days 1-2 mm hypocotyl sections were excised and soaked 2 hours in overnight-grown *Agrobacterium* diluted 1:20 in liquid MS. Alternatively, hypocotyl sections were soaked 2 hours in undilute overnight-grown *Agrobacterium*, then blotted gently. The sections were placed on non-selective medium (MS with 10 µg/ml BAP and 1 µg/ml NAA). After 2 days cocultivation in light, flax sections were transferred on MS with, in addition to NAA and BAP, 100 µg/ml kanamycin and 400 µg/ml cefuroxime for selection of transformed tissue and counterselection against freeliving *Agrobacteria*. Callus formation was followed by dark green shoot formation. These shoots could be readily propagated on the same medium and induced to regenerate roots on MS containing 0.05 µg/ml kinetin.

2.3.1 Selection

The presence of the transformed genes was selected by kanamycin resistance, on medium containing 100 µg/ml kanamycin sulphate. Small amounts of plant material on this medium cannot usually grow unless *nptII* is integrated. Potential transgenotes were then screened for luminescence and Southern blots performed on DNA from luminescent material.

Apparent transgenic plantlets or calli were screened for luminescence, usually by means of a luminometer (Langridge et al., 1989; 1991), provided by Dr. A.A. Szalay. Various plant tissues were excised, weighed, and placed in Eppendorf tubes to which 0.5 ml of lux buffer (50 mM Na_2HPO_4, pH 7.0, 50 mM β-mercaptoethanol, 0.4 M sucrose; Langridge et al., 1991), was added and the contents broken up and ground by means of a fitted plastic pestle. The tube contents were then transferred to a glass scintillation vial which was placed in the counting chamber of a Turner TD-20e luminometer and left for about 15 seconds while residual fluorescence decreased. The chamber was fitted with a port closed with a rubber septum through which a 1 ml syringe with 0.5-0.6 ml of FMN-tricine buffer (100 µM $FMNH_2$, reduced by white light, in 200 mM tricine, pH 7.0; Langridge et al., 1991) and 20 µl of 0.1% *n*-decanal was placed. The photon counter was activated and after a brief interval the contents of the syringe rapidly injected. Light units (1 LU=1.6×10^6 photons) were recorded. 5 LU was taken as a minimal measurement for lux^+ status.

2.4 DNA isolation, Southern blotting, and hybridization

DNA was isolated from luminescent material to test the presence of P450 genes by Southern blotting. The method of Dellaporta et al. (1985) as modified by Dr. A. Good was used on a small or large scale (Appendix 1). Tissues were ground, cell walls ruptured with SDS, debris spun down and DNA precipitated from the supernatant.

For Southern blots, 10-20 µg of plant DNA was digested overnight with 3-5 units/µg *Eco*RI. RNase A (0.1 mg/ml) was added to the digestion mixture to remove RNA. If necessary, digested DNA was concentrated by ethanol precipitation. DNA was run on 0.7% agarose gels for several hours at suitable voltages. DNA from #5 tobacco (SR1 transformed with pPCV701*lux*A/*lux*B, from a very bright plant) served as a negative control. DNA from the same #5 plant was mixed with 1-2 ng of plasmid DNA carrying the P450 gene in question for use as a positive control

Blotting in most cases followed the capillary adsorption technique of Southern (Draper et al., 1988). For a few gels a vacuum blot apparatus (BioRad Model 785), provided by Dr. A. Good, was used. Gels were blotted onto Gene-Screen Plus (NEN) following the manufacturer's instructions. Gels were usually treated with 0.25 M HCl to nick and facilitate transfer of larger DNA fragments. They were then denatured, neutralized, and blotted overnight. For vacuum blotting manufacturer's instructions were followed. Blots were dried and stored in filter paper folders until hybridization.

^{32}P-labelled probes were prepared by the oligolabelling method. 25-50 ng of purified P450 fragment was oligolabelled for each membrane. The level of labelling was tested by counting acid-precipitable counts of an aliquot. Specific activity of probes ranged from 3×10^8 to 1.2×10^9 counts per minute per µg DNA. Since a very large amount of plant DNA is present relative to the amount sought by the probe, the highest possible levels of labelling were desired.

Prehybridization, hybridization and washing took place according to NEN suggestions. Hybridization was overnight at 42^{o}C in hybridization solution including 1 M NaCl, 1% sodium dodecyl sulphate (SDS), 50% formamide, and 10% dextran sulfate. After washing, membranes were folded in clear Saran wrap, and appressed to Fuji X-ray film in film cassettes. They were exposed at -70^{o}C from 10 hours to 7 days according to signal intensity, then developed.

2.5 Expression of P450 genes

Transcription of P450 genes was examined by Northern and slot blotting, translation by Western and protein slot blotting, enzyme activity by assays of microsome and crude cell extract function and phenotypic effects by growth of seedlings from self-fertilized plants.

2.5.1 RNA isolation and blotting

The high content of native RNase in plant tissue makes RNA isolation difficult. Standard methods were used to decontaminate lab equipment used to isolate RNA (Slater, 1991). The RNA isolation method of Yeh et al. (1991) was most frequently used. This method involves grinding the cells in a high-Sarkosyl buffer followed by phenol:chloroform extractions and isopropanol precipitation. For Northern blots it was modified by the addition of Macaloid (National Lead Company, Houston, Texas) prepared according to Sambrook et al. (1989) and added to the grinding buffer. Slowness in preparation, prolonged storage even at -70^{o}C, or less than rigorous RNase decontamination gave degraded RNA. Where storage was necessary, storage in isopropanol at -70^{o}C preserved RNA best.

For Northern blots tobacco RNA was enriched in mRNA by use of an oligo (dT) cellulose column (Kingston, 1993). Tobacco and flax RNA was denatured and run on formaldehyde-MOPS (3-[N-morpholino]propanesulfonic acid) denaturing gels (1.0-1.2% agarose) according to Sambrook et al. (1989), without ethidium bromide. BRL RNA markers were run on one lane which was cut off, stained with ethidium bromide (0.5 µg/ml in 0.1 M ammonium acetate and 2 mM $MgCl_2$) and destained overnight. The rest of the gel was capillary blotted onto supported nitrocellulose (Schleider and Schull) and vacuum-baked for 1 hour at 80^{o}C. ^{32}P-labelled probes were prepared as for Southern blots, using *Cyp* or *lux*F DNA fragments. Unincorporated nucleotides were removed on a BioGel P30 (BioRad) or Sephadex G-50 (Pharmacia) column. Hybridization and washes were performed at 65^{o}C according to Sambrook et al. (1989), using Denhardt's reagent. Membranes were exposed to film for suitable times at -70^{o}C.

2.5.2 Crude cell extract and microsome preparation

Crude cell extracts and microsome samples were prepared according to Reichhart et al. (1980) or Zimmerlin et al. (1992). These methods differ chiefly in buffer composition. For P450IIA5 samples, when activity was not to be assayed, 100 µM coumarin was added to all solutions; this is reported to stabilize the enzyme (Juvonen et al., 1988). Protease inhibitors leupeptin and pepstatin were sometimes added to isolation buffers at 5 µg/ml. Fresh plant material was thoroughly ground in 3-4 volumes of buffer in chilled mortars and centrifuged at 10,000 g for 10 minutes at 4^{o}C. Samples of supernatant were used as crude cell extract. Supernatant was also spun 1 hour at 100,000 g in a fixed-angle or swing-bucket rotor to yield a microsome pellet, which was resuspended in a small volume of freezer buffer (Reichhart et al., 1980; Zimmerlin et al., 1992). For assays of enzyme function, extracts were used fresh if possible. Amounts of protein were determined according to Bradford (1976). Crude extracts contained 2.0-2.5 mg protein per ml; microsomal pellets contained 3-5 mg protein/ml.

2.5.3 Western blots

Microsome and crude cell extracts were incubated in cholate buffer at 30-37^{o}C for 60 minutes to solubilize membrane proteins (Reichhart et al., 1980). They were then denatured by treatment with SDS at 95^{o}C and loaded onto 7-15% polyacrylamide denaturing gels (Sambrook et al., 1989). Each lane was loaded with 1-5 mg protein. Gels were run overnight at 30 V. Part of each gel was cut off and stained with Coomassie blue dye, then destained. The remainder was electroblotted onto nitrocellulose as suggested by BioRad, using a BioRad blotting apparatus overnight at 30V followed by 1 hour at 60^{o}C. The blotted gel was then stained with Coomassie blue dye to estimate the degree of transfer.

The nitrocellulose membrane was probed with antibody to P450IA1. Membranes were blocked with 1% bovine serum albumen and washed with TBST (TBS+0.05% Tween 20) following Promega suggestions. Primary antibody was anti-P450IA1 monoclonal provided by Dr. D. Pompon. Secondary antibody was affinity-purified alkaline phosphatase conjugated goat anti-mouse IgG from BRL. All antibodies were diluted according to their maker's suggestion. Antibody incubations and washes with TBST all followed standard practice. Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) colour reagents were used as suggested by ProMega and allowed to develop overnight in the dark. In some cases the colour reaction was later enhanced by increasing both colour reagents 3-fold and allowing a further 4-8 hours incubation. Colour development was ended by washing in water and the membranes were stored damp at 4°C until photographed.

2.5.4 Assays of enzyme activity

For fluorometric assays, activity of crude cell extract or microsome samples was measured in a Perkin-Elmer LS50 fluorometer. P450IA1 enzyme activity was assayed by conversion of 7-ethoxyresorufin to resorufin, using a slightly modified protocol of Dr. D. Pompon (personal communication). In a fluorometer cuvette were combined 2-100 µl of crude cell extract or microsome fraction, 6 µl of 0.45 mM 7-ethoxyresorufin in ethanol, 16 µl of 10 mg/ml NADPH, freshly prepared, and 3 ml of buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA). The substrate or NADPH could be added during measurement. The product was measured by fluorescent emission at 586 nm from excitation at 530 nm over a 90-second period. Commercial S9 fraction (MolTox) was used as a positive control. S9 is microsomal fraction of liver prepared from Sprague-Dawley rats with P450 genes induced by Aroclor, a mixture of various benzene derivatives; it is usually used as a representation of liver function in the Ames assay for mutagenicity. P450IA1 is present in this fraction.

P450IIA5 activity was assayed by conversion of coumarin to its 7-hydroxylated product umbelliferone, either according to Kaipainen et al. (1985), or more frequently by a slight modification of Pompon's assay, using coumarin instead of 7-ethoxyresorufin. Umbelliferone was measured by fluorescent emission at 440 nm from excitation at 390 nm. No positive control was available short of getting custom S9 fraction from particular mouse strains (rats lack significant coumarin hydroxylase activity), so none was used.

To examine the possibility of protein phosphorylation, 0.9 ml of crude cell extract was incubated for 30 minutes prior to assay in 2-20 units of calf intestinal phosphatase.

To test that the plant preparations were able to support P450 activity, the activity of cinnamate 4-hydroxylase (conversion of *t*-cinnamate to *p*-coumarate), an activity presumed to exist in all vascular plants, was measured. To induce plant P450 enzymes, transformed shooty flax was grown in medium containing 0.1% yeast extract, or UV light was administered daily for 5-10 minutes for about 7 days. Transformed tobacco suspension was grown in medium containing 0.1% yeast extract or 25 mM $MnCl_2$, or UV light was administered once for 30 minutes with cells spread in a shallow layer in Petri dishes. The brightest available tobacco suspension cultures were used; the flax emitted little light. Microsome and crude cell extracts were made from these induced tissues.

Crude cell extracts containing about 0.2 mg protein were added to a reaction mixture (50 mM Tris-HCl, pH 7.5; 1 mM EDTA; 2.3 µM *t*-cinnamate; 1 mg/ml NADPH) and incubated for several hours at room temperature. Samples were taken and stopped with 5N HCl at different times, then stored at -10°C until measurement. *P*-coumarate formation was examined by UV scanning. Equal volumes of aqueous samples were mixed with methanol and UV absorbance from 250-330 nm was traced, using the time 0 sample as a control.

2.5.5 Seedling growth

Seed from self-fertilized transgenic tobacco plants was germinated and grown on MS salts plates. Individual seedlings, 5-10 mm tall, were transferred to 35x100 mm tubes containing about 3 cm sterile vermiculite and 12 ml MS salts. These were sealed with Saran wrap and elastic bands to prevent dessication and placed in every second row in test tube racks. When the seedlings were established, they were paired for size and one of each pair treated with coumarin in dimethylsulfoxide (DMSO), the other with DMSO alone. Their height (greatest distance from root/shoot juncture) was measured about once a week. They were supplemented at intervals with more coumarin in DMSO or DMSO and MS salts as their growth rate and appearance seemed to warrant. Trials were ended when the tallest seedlings were about 7 cm tall. At this point it was not known which of the seedlings in the heterogeneous population carried the P450 gene. The status of the lux^+ or lux^- allele was taken as its indicator. Eight mm discs were punched from seedling leaves and placed on wet filter paper discs containing 0.5 μg/ml 2,4-D overnight at 24^oC. Luciferase activity was then measured by luminometer. Correlations between luciferase status and growth rates were drawn.

3. Results and Discussion

3.1 Effects of phenolic compounds and weed extracts on crop species

Before studying the effects of altering the genetic properties of crops, it was necessary to know the levels of injury produced by common allelochemicals, how they act in combination, as they occur in the field, what level of injury was produced by weed extracts under the same experimental conditions, and whether there was any correspondence between level of injury by allelochemicals under experimental conditions and level of interference by weeds in the field.

3.1.1 Flax

The largest selection of phenolic compounds was tested on flax cv. McGregor. They included the phenolic compounds implicated in wild oat allelopathy or crop residue effects, and a few relatives of these. Results appear in Table 5 and Figure 8.

Of phenolic acids tested, none inhibit germination. All reduce shoot and root length and dry weight severely at the higher doses, most at the lower doses also. The order of toxicity, from most to least severe, is approximately: ferulate, *p*-coumarate, *t*-cinnamate, *p*-hydroxybenzoate, vanillate, syringate, caffeate. Lesions were often qualitatively distinct. For example, caffeic acid produced some root necrosis, while ferulic acid produced reddish or darkened lower stems. The patterns of damage were distinctive enough that it was possible to identify treatment and dose by looking at the roots in many cases. Phenolic acids produce an initial pH as low as 3.4. However, controls at this initial pH (unbuffered) appeared uninjured in flax and other crops.

The coumarins caused more severe injury than the phenolic acids (Table 5; Figure 8B). Coumarin reduced shoot and root length very severely at lower doses and inhibited germination completely at 4 mM. The other coumarins appear slightly more toxic than the phenolic acids. They did not inhibit germination. Umbelliferone appears slightly more toxic than esculetin or scopoletin. Coumarins produce distinct patterns of root injury; esculetin produced necrotic spots, while umbelliferone produced thick blunt roots.

Generally, the degree of toxicity is the same as the degree of hydrophobicity, as was observed by Glass and Dunlop (1974). Coumarin is most hydrophobic and by far the most toxic of the compounds tested. Those with polar side groups appeared less toxic.

Notable in experiments dealing with weed extracts is the variability in the weeds. Canada thistle shoot extract, for example, may inhibit flax shoot growth almost entirely or very little. Table 6 shows that different lots of Canada thistle or wild oats produce this kind of variable response for most parameters. Possible factors behind this variability in the weed extracts include age and developmental stage of the weed, soil, degree of crowding, exposure to herbicides, season and weather conditions, weed genotype, and preparation conditions. Extraction in boiling water has some effect on the toxicity of the preparation, but this effect is not consistent. There is likely to be a balance between extraction and destruction of factors by heat. None of these sources of variability was further examined. If this range of effect is typical for weeds, however, the non-repeatability of experiments involving weed residues and extracts may be accounted for. Determination of the conditions which produce particularly toxic or less toxic weeds would be valuable.

These caveats in place, it appears that flax is generally affected by the weed extracts used, in some cases severely. Germination is little affected, but wild oat and Canada thistle shoot and root extracts may reduce both shoot and root extension severely. Figure 9 shows the magnitude of damage to flax roots which can be caused by wild oat extracts. Typically roots are more or less stunted; the main root is very short and

Table 5. Effects of phenolic compounds on flax seedlings at 14 days.

Expt.	Treatment	Germination	Shoot length			Root length			Shoot dry wt.		Root dry wt.	
#		%	mm	s.e.	% control	mm	s.e.	% control	mg	% control	mg	% control
120	Control	100	76.3	2.2	100	126.3	4.1	100	7.8	100	4.2	100
	ferulic 10-4 M	95	68.5	3.0	90	133.1	4.7	105	7.3	93	4.4	103
	10-3 M	100	50.1	2.4	66	150.0	6.5	119	5.7	74	3.6	85
	4x10-3 M	95	29.2	0.8	38	33.0	4.0	26	3.9	50	1.6	37
	p-coumaric 10-4 M	100	72.2	2.3	95	131.3	5.0	104	7.9	100	4.4	105
	10-3 M	100	62.2	2.6	81	111.9	3.9	89	6.6	85	4.2	99
	4x10-3 M	95	30.7	1.5	40	39.9	6.9	32	4.2	54	2.0	48
	p-hydroxybenzoic											
	10-4 M	100	73.7	2.3	96	104.4	3.2	83	7.8	101	4.7	110
	10-3 M	95	50.9	1.9	67	86.9	3.5	69	5.8	75	3.3	77
	4x10-3M	100	34.2	1.1	45	62.5	3.5	50	3.8	49	2.2	51
	vanillic 10-4 M	95	60.9	5.1	80	111.9	2.8	89	6.9	89	4.5	105
	10-3 M	95	50.1	1.2	66	63.1	2.8	50	5.4	69	4.3	101
	4x10-3 M	100	32.8	1.1	43	32.9	2.0	26	4.1	52	3.2	75
119	Control	90	78.2	1.3	100	111.9	3.3	100	7.8	100	3.9	100
	ferulic 10-4 M	95	71.4	3.3	91	124.4	6.3	111	7.4	95	3.8	98
	10-3 M	100	56.2	1.9	72	118.8	4.3	106	5.5	71	3.2	82
	4x10-3 M	100	30.9	0.9	40	34.4	5.0	31	3.7	47	1.7	43
	p-coumaric 10-4 M	100	70.2	2.5	90	110.6	5.3	99	7.1	92	4.1	105
	10-3 M	100	60.4	2.5	77	106.3	4.0	95	6.0	77	3.9	101
	4x10-3 M	100	28.3	1.0	36	21.3	2.4	19	3.2	41	1.5	40
	p-hydroxybenzoic											
	10-4 M	100	70.1	2.0	90	96.8	4.3	86	7.1	91	4.4	115
	10-3 M	100	52.7	1.2	67	63.8	4.4	57	5.3	69	2.7	70
	4x10-3M	95	34.6	1.3	44	53.9	4.9	48	3.6	46	2.0	51
	vanillic 10-4 M	100	66.3	2.3	85	106.9	5.3	96	6.7	87	4.3	112
	10-3 M	95	50.5	1.9	65	54.5	1.8	49	5.3	68	3.7	95
	4x10-3 M	100	36.1	0.9	46	27.6	2.0	25	3.8	49	2.4	63
	pH 3.4 control	90	75.3	1.6	96	123.8	4.4	111	7.3	93	4.4	113

Table 5 continued.

	Treatment	Germination	Shoot length			Root length			Shoot dry wt.		Root dry wt.	
		%	mm	s.e.	% control	mm	s.e.	% control	mg	% control	mg	% control
51	Control	92	90.7	6.1	100	90.7	n.d.	100	7.7	100	2.2	100
	syringic 10-4 M	100	101.7	11.6	112	85.1		94	9.9	128	3.2	145
	10-3 M	100	101.1	11.6	111	84.5		93	9.5	123	3.3	150
	4x10-3 M	100	60.3	5.9	66	60.3		66	6.3	81	2.3	105
	t-cinnamic 10-5 M	100	106.3	3.1	117	106.3		117	8.9	115	2.4	110
	10-4 M	100	102.3	4.0	113	102.3		113	8.9	116	2.8	127
	10-3M	100	72.6	5.5	80	72.6		80	6.3	82	2.3	105
82	Control	98	95.7	2.7	100	153.1	n.d.	100	12.6	100	6.1	100
	caffeic 10-4 M	100	91.0	7.3	95	143.8	13.4	94	10.9	87	4.8	79
	10-3 M	91	95.2	3.1	100	141.9	5.6	93	10.7	85	5.5	90
	2x10-3 M	92	82.8	5.8	87	91.3	7.0	60	8.8	70	4.8	79
	4x10-3 M	100	75.0	3.0	78	85.0	3.4	56	7.5	60	4.1	67
122	Control	100	82.0	1.4	100	126.3	4.6	100	7.7	100	4.2	100
	coumarin 10-4 M	95	53.2	3.2	65	56.6	3.1	45	4.8	62		
	10-3 M	85	7.1	0.6	9	<5		<5				
	4x10-3 M	0										
	esculetin 10-4 M	95	74.8	4.7	91	118.1	4.1	94	6.4	83	3.9	93
	10-3 M	90	58.0	3.7	71	99.4	3.2	79	5.2	68	3.4	80
	4x10-3 M	95	34.8	1.9	42	63.5	5.9	50	3.6	47	2.1	49
	scopoletin 10-4	95	71.8	4.8	88	105.0	4.7	83	7.1	92	4.1	97
	10-3 M	90	53.1	3.5	65	81.9	3.7	65	5.2	68	3.3	78
	4x10-3	90	40.1	2.1	49	41.0	2.6	32	3.7	47	2.4	56
	umbelliferone											
	10-4 M	95	65.8	2.8	80	101.3	6.0	80	6.2	80	4.4	104
	10-3 M	95	40.1	1.8	49	27.5	4.1	22	4.3	55	2.3	55
	4x10-3 M	90	32.5	2.9	40	26.3	2.2	21	4.2	54	2.3	56

A

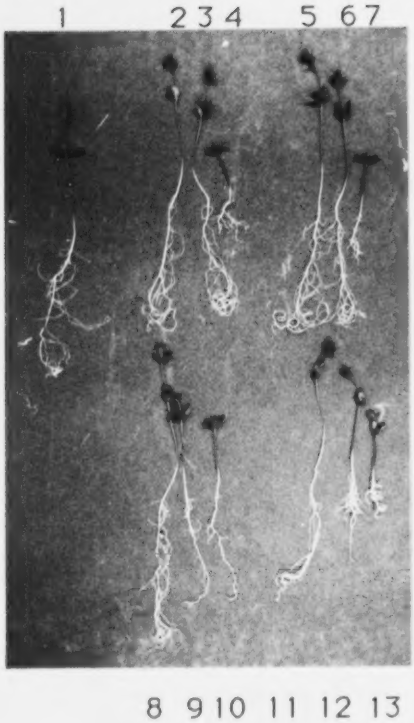
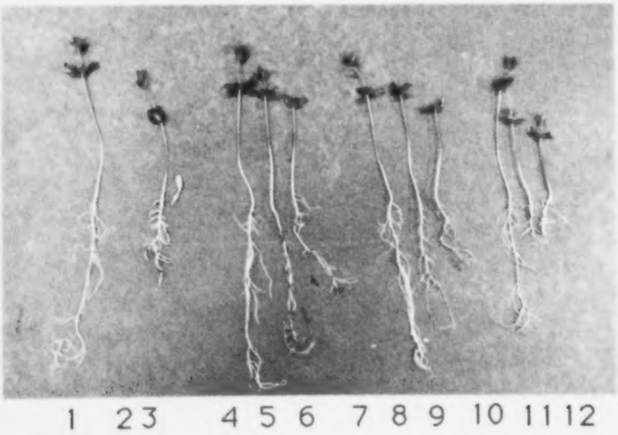


Figure 8. Effects of phenolic compounds on flax. Approximately 0.3X.
A, phenolic acids.
1, control;
2, 0.1 mM ferulate;
3, 1 mM ferulate;
4, 4 mM ferulate;
5, 0.1 mM *p*-coumarate;
6, 1 mM *p*-coumarate;
7, 4 mM *p*-coumarate;
8, 0.1 mM *p*-hydroxybenzoate;
9, 1 mM *p*-hydroxybenzoate;
10, 4 mM *p*-hydroxybenzoate;
11, 0.1 mM vanillate;
12, 1 mM vanillate;
13, 4 mM vanillate.

B, coumarins. 1, control; 2, 0.1 mM coumarin; 3, 1 mM coumarin; 4, 0.1 mM esculetin; 5, 1 mM esculetin; 6, 4 mM esculetin; 7, 0.1 mM scopoletin; 8, 1 mM scopoletin; 9, 4 mM scopoletin; 10, 0.1 mM umbelliferone; 11, 1 mM umbelliferone; 12, 4 mM umbelliferone.

B



twisted. Canada thistle extracts produced this effect very markedly. The root distortions may result from interactions of the compounds in weed extracts with the normal flax phytohormones. Cotyledons were generally somewhat bleached and hypocotyls darkened; these are probably general symptoms of stress. Stinkweed extract is intermediate in effect on shoot height, but more severe in effect on root length than extracts of wild oats or Canada thistle. However, only one sample of stinkweed was tested. Generally, Canada thistle extracts are more severe in their effect than those of wild oats.

3.1.2 Wheat

Nearly all the same compounds were tested on wheat cv. Katepwa. Results appear in Table 7 and Figure 10. 1 mM of *t*-cinnamic acid slightly reduced germination; other phenolic acids did not. Shoot length was slightly reduced at the highest doses of most phenolic acids, and root length was severely reduced. At almost every point the injury was less than that seen in flax. Some qualitative marks of injury were noted, but these were much less marked than in flax. The order of toxicity, from most to least severe, is approximately: *p*-coumarate, syringate, ferulate, vanillate, *t*-cinnamate, *p*-hydroxybenzoate.

Of the coumarins, coumarin reduced germination to 20% at 1 mM and 0% at 4 mM (Table 7). Scopoletin at 4 mM slightly reduced germination; esculetin and umbelliferone did not. Shoot length was affected, particularly by coumarin, but much less than shoot length in flax. Root length was more severely reduced than in flax. Wheat therefore appears to respond differently to coumarins than to phenolic acids, relative to flax. Coumarins produced characteristic "signatures" of injury on wheat roots. Umbelliferone or coumarin made them yellow or brown and conspicuously thickened; scopoletin and esculetin produced necrotic spots or patches. Coumarin was the most toxic overall, followed by umbelliferone, scopoletin and esculetin. Again, the degree of hydrophobicity corresponds fairly well with the degree of toxicity.

All the cautions noted in looking at the effects of weed extracts on flax apply equally to wheat. The same extracts were tested on flax and wheat, generally in parallel experiments, so that effects of particular lots of weed extract on flax and wheat can be compared with relative confidence.

Wild oat extracts appear to have little or no effect on wheat seedling shoot length (Table 8). Overall wild oat damage was less than on flax. In particular, there was less shoot damage. With Canada thistle, however, germination, shoot height and root length were severely affected by some extracts, about as much as flax.

Stinkweed had more severe effects on wheat than did some lots of Canada thistle and wild oat. However, these lots of wild oat and Canada thistle extract were much less toxic than others of the same species. Therefore results from this single sample of stinkweed should be treated with caution.

3.1.3. Oats

Limited experiments on oat cvs. Cascade and Dumont were performed very much like those on wheat. Results appear in Table 9. *P*-coumaric acid produced similar effects in both species; oats grew better but not substantially so. If logarithmic interpolation is correct, scopoletin affected oat shoot less than wheat shoot, but oat root more than wheat root. The difference between wheat shoot and Dumont oat shoot, and the difference between wheat root and Cascade oat root, is substantial. Repetition of the experiment using the same molarity of scopoletin would be useful to refine this observation. Possibly the interaction between scopoletin and IAA differs in oats and wheat. Oat seedlings normally release scopoletin; wheat seedlings do not (Rademacher and Martin,

Table 6. Effects of weed extracts on flax seedlings at 14 days.

Expt.	Treatment	Germination	Shoot length			Root length			Shoot dry wt.		Root dry wt.	
#		%	mm	s.e.	% control	mm	s.e.	% control	mg	% control	mg	% control
220	Control	85	101.6	3.4	100	100.5	5.9	100	9.2	100	2.2	100
	stinkweed	80	92.3	3.6	91	75.9	4.7	76	10.0	108	3.0	136
	Canada thistle	85	95.9	2.3	94	95.0	4.3	90	11.0	120	3.0	136
	wild oats	90	87.3	3.9	86	73.7	4.5	73	9.7	105	2.3	105
103	Control	95	99.8	1.4	100	128.8	3.4	100	9.3	100	3.9	100
	wild oat shoot	90	86.2	3.2	86	125.6	9.3	98	7.7	83	4.2	106
	wild oat root	100	86.7	4.1	87	92.5	7.8	72	8.7	93	4.3	109
24	Control	100	118.9	1.7	100	n.d.			13.1	100	3.3	100
	wild oat shoot	100	33.2	12.3	28				6.3	48	1.3	39
	same, boiled	100	43.7	5.3	37				6.6	50	1.8	52
	wild oat root	83	79.8	9.2	67				7.6	58	2.5	75
	same, boiled	100	72.3	16.9	61				7.3	56	2.1	63
30	Control	100	105.0	3.4	100	n.d.			10.3	100	2.1	100
	thistle shoot	50	8.0	3.1	8							
	same, boiled	67	21.0	15.1	20				5.6	54	2.3	110
	thistle root	100	80.4	10.2	77				7.7	75	3.0	143
	same, boiled	100	52.7	13.1	52				6.1	59	1.8	86

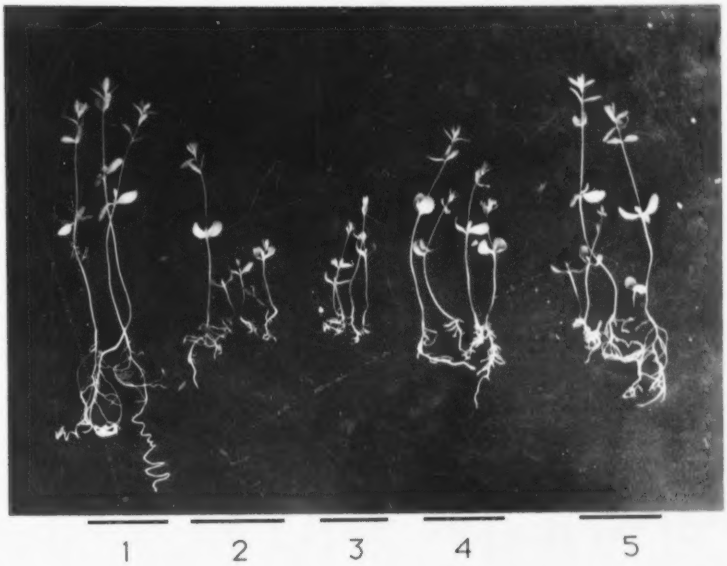


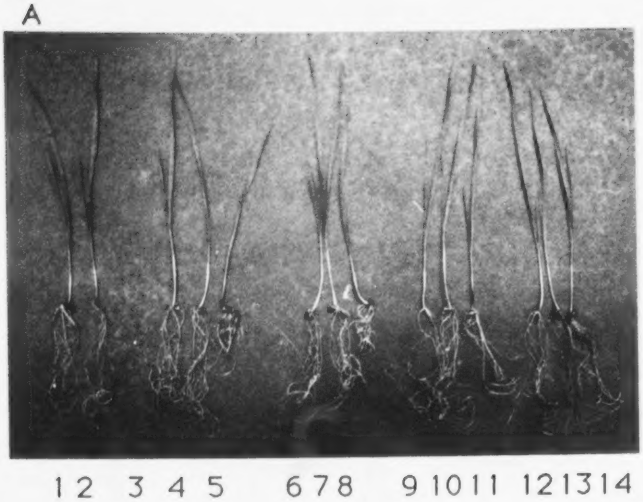
Figure 9. Effects of wild oat extracts on flax. Approximately 0.5X.
1, control;
2, wild oat shoot extract, unboiled;
3, wild oat shoot extract, boiled;
4, wild oat root extract, unboiled;
5, wild oat root extract, boiled.

Table 7. Effects of phenolic compounds on wheat at 10 days.

Expt.	Treatment	Germination	Shoot length			Root length			Shoot dry wt.		Root dry wt.	
#		%	mm	s.e.	% control	mm	s.e.	% control	mg	% control	mg	% control
102	Control	85	184.6	9.0	100	161.9	7.1	100	16.7	100	27.2	100
	ferulic 10-4 M	95	190.1	8.1	103	168.8	6.1	104	16.6	99	22.6	83
	10-3 M	90	181.7	10.3	98	145.6	8.3	90	16.1	96	23.8	88
	4x10-3 M	100	158.5	8.1	86	76.2	5.3	47	14.6	88	19.0	70
	p-coumaric 10-4 M	90	185.8	11.2	101	169.3	6.0	105	17.6	105	27.7	102
	10-3 M	95	173.5	12.7	94	146.3	8.5	90	14.0	84	22.4	82
	4x10-3 M	90	143.6	9.5	78	65.0	4.6	40	14.0	84	18.5	68
	p-hydroxybenzoic											
	10-4 M	95	191.8	8.4	104	155.6	7.4	96	17.0	102	25.8	95
	10-3 M	95	184.9	9.7	100	178.8	6.9	110	14.6	88	22.7	84
	4x10-3M	100	177.5	8.7	96	164.3	6.1	102	13.0	78	22.9	84
	vanillic 10-4 M	95	197.2	5.8	107	158.1	6.5	98	17.9	107	26.6	98
	10-3 M	89	193.3	9.9	105	135.6	4.9	84	18.4	110	29.7	109
	4x10-3 M	100	177.8	8.9	96	101.3	4.8	63	16.2	97	24.9	91
116	Control	85	168.3	8.9	100	156.3	6.2	100	17.3	100	30.0	100
	ferulic 10-4 M	85	182.7	3.8	109	168.1	6.7	108	17.2	99	32.5	108
	10-3 M	95	168.5	6.1	100	161.3	5.4	103	15.6	90	27.3	91
	4x10-3 M	90	137.4	5.5	82	66.7	5.9	43	13.3	76	20.3	68
	p-coumaric 10-4 M	100	169.7	5.4	101	168.1	4.1	108	16.5	95	31.6	105
	10-3 M	90	156.6	7.4	93	151.7	4.3	97	13.1	75	23.5	78
	4x10-3 M	90	121.9	7.0	72	43.4	3.0	28	12.3	71	19.8	66
	p-hydroxybenzoic											
	10-4 M	90	172.5	10.0	103	164.4	5.6	105	15.2	87	27.6	92
	10-3 M	95	156.7	10.0	93	179.4	4.9	115	13.5	78	24.3	81
	4x10-3M	95	159.8	5.6	95	145.0	6.7	93	13.8	80	25.5	85
	vanillic 10-4 M	95	167.3	9.3	99	154.4	2.6	99	16.6	96	30.2	101
	10-3 M	95	165.6	5.4	98	126.9	2.8	81	15.6	90	31.2	104
	4x10-3 M	85	147.1	10.1	87	102.5	4.9	66	13.2	76	25.2	84
	pH 3.8 control	95	164.3	9.0	98	165.0	4.5	106	16.3	94	29.8	99

Table 7 continued.

Expt.	Treatment	Germination	Shoot length			Root length			Shoot dry wt.		Root dry wt.	
#		%	mm	s.e.	% control	mm	s.e.	% control	mg	% control	mg	% control
54	Control	92	199.5	13.3	100	n.d.			20.4	100	30.2	100
	syringic 10-4 M	92	193.2	17.6	99				20.5	101	28.5	95
	10-3 M	100	214.5	6.9	107				19.5	96	21.6	72
	4x10-3 M	92	148.9	18.2	75				15.5	76	23.2	77
	t-cinnamic 10-5 M	100	192.6	14.2	97				18.0	88	25.4	84
	10-4 M	100	222.0	11.5	111				21.0	103	29.9	99
	10-3M	67	193.1	7.2	97				16.6	81	24.4	81
101	Control	95	185.5	9.8	100	137.9	5.4	100	19	100	23	100
	coumarin 10-4 M	95	148.7	8.4	80	28.9	2.2	21	16	85	13	58
	10-3 M	20	24.5	11.4	13	3.0	2.4	2				
	4x10-3 M	0										
	esculetin 10-4 M	95	186.7	10.9	101	143.1	7.6	104	17	91	22	96
	10-3 M	95	173.7	13.0	94	101.3	7.3	73	17	87	18	79
	4x10-3 M	95	159.6	9.1	86	48.8	4.5	35	17	89	17	75
	scopoletin 10-4	95	179.8	14.1	97	126.9	8.1	92	19	101	23	100
	10-3 M	95	194.0	9.1	105	86.3	7.5	62	17	91	22	96
	4x10-3	80	150.1	13.4	81	44.1	3.5	32	15	77	16	71
	umbelliferone											
	10-4 M	100	162.3	8.0	87	37.5	4.2	27	17	87	13	58
	10-3 M	95	120.2	11.3	65	15.5	1.1	11	14	75	18	78
	4x10-3 M	95	128.3	8.7	69	11.6	0.9	8	14	74	17	76



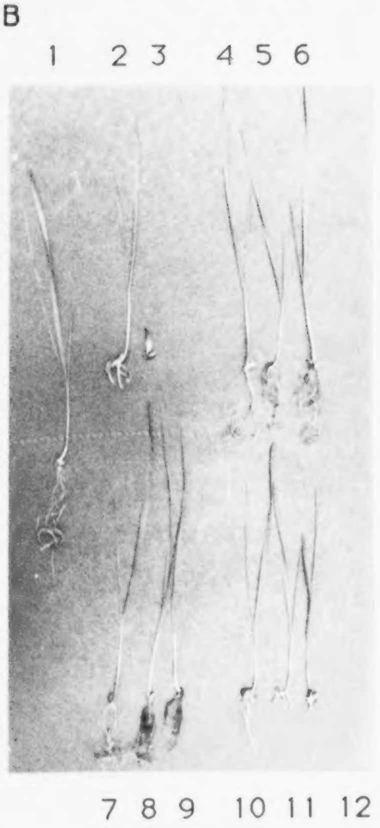


Figure 10. Effects of phenolic compounds on wheat. Approximately 0.3X. A, phenolic acids. 1, control; 2, pH control; 3, 0.1 mM ferulate; 4, 1 mM ferulate; 5, 4 mM ferulate; 6, 0.1 mM *p*-coumarate; 7, 1 mM *p*-coumarate; 8, 4 mM *p*-coumarate; 9, 0.1 mM *p*-hydroxybenzoate; 10, 1 mM *p*-hydroxybenzoate; 11, 4 mM *p*-hydroxybenzoate; 12, 0.1 mM vanillate, 13, 1 mM vanillate; 14, 4 mM vanillate. B, coumarins. 1, control; 2, 0.1 mM coumarin; 3, 1 mM coumarin; 4, 0.1 mM esculetin; 5, 1 mM esculetin; 6, 4 mM esculetin; 7, 0.1 mM scopoletin; 8, 1 mM scopoletin; 9, 4 mM scopoletin; 10, 0.1 mM umbelliferone; 11, 1 mM umbelliferone; 12, 4 mM umbelliferone.

Table 8. Effects of weed extracts on wheat seedlings at 10 days.

Expt.	Treatment	Germination	Shoot length			Root length			Shoot dry wt.		Root dry wt.	
#		%	mm	s.e.	% control	mm	s.e.	% control	mg	% control	mg	% control
220	Control	75	193.3	13.7	100	150.2	6.4	100	21	100	18.2	100
	stinkweed	90	176.8	12.7	91	140.8	4.9	94	18.5	88	18.5	102
	Canada thistle	90	190.0	9.1	98	150.1	5.3	100	21	101	18.8	103
	wild oats	100	191.1	10.4	99	136.8	5.4	91	20	96	19	102
103	Control	100	215.7	6.0	100	138.1	4.8	100	n.d.		n.d.	
	wild oat shoot	80	183.8	14.2	85	158.8	2.3	115				
	wild oat root	95	215.0	5.4	100	130.6	5.9	95				
25	Control	100	169.3	24.7	100	n.d.			18	100	n.d.	
	wild oat shoot	83	162.3	52.3	96				14	78		
	same, boiled	83	181.0	30.9	107				16	89		
	wild oat root	83	198.8	14.1	117				20	111		
	same, boiled	83	217.0	17.8	128				24	133		
30	Control	100	169.8	20.3	100	n.d.			17.4	100	28.9	100
	thistle shoot	17	31.0		18				2.5	14	27	93
	same, boiled	20	5.0		3							
	thistle root	50	204.0	44.0	120				14.4	82	26	90
	same, boiled	67	65.5	36.8	39				6.2	36	29.3	101

Table 9. Effects of phenolic compounds on Cascade and Dumont oats at 10 days.

Expt. #	Treatment	Germination %	Shoot length mm	s.e.	% control	Root length mm	s.e.	% control	Shoot dry wt. mg	% control	Root dry wt. mg	% control
97	Cascade											
	Control	85	153.2	10.7	100	166.6	8.3	100	15.0	100	n.d.	
	p-coumaric acid 10-3 M	85	152.1	9.8	99	162.3	7.7	97	14.9	99		
	scopoletin 5x10-4 M	65	144.0	9.5	94	63.4	5.3	38	12.7	85		
	Dumont											
	Control	95	178.9	6.2	100	179.4	4.3	100	17.4	100	n.d.	
	p-coumaric acid 10-3 M	85	168.1	7.4	94	193.0	4.3	108	16.2	93		
	scopoletin 5x10-4 M	95	188.2	3.8	105	97.0	8.1	54	18.0	103		

1957; Fay and Duke, 1977).

Very limited trials of weed extracts on oats and wild oats are not shown. They were little affected.

3.1.4 Barley

Limited tests of phenolic compounds on barley were performed on four cultivars; results appear in Table 10.

Barley treated with *p*-coumaric acid appears more severely affected than oats or wheat at the same dose. Most damage occurs in the root. Barley responded to scopoletin much more like oats than like wheat. That is, shoots were affected relatively less and roots more. However, the limited examination of barley makes detailed comparisons hazardous.

Effects of weed extracts on barley appear in Tables 10 and 11. Compared to flax and wheat, injury is slight. It appears that shoots are unaffected, but roots are shortened and thickened.

3.1.5 Tobacco

Prior to the introduction of detoxifying genes into tobacco, the effects of several phenolic compounds on tobacco germination were determined. Effects of ferulic acid on some seedling growth parameters were also examined. The goal was to find whether its degree of tolerance to allelochemicals was in the same general range as the other species tested.

Results of germination assays of tobacco cv. SR1 on agar appear in Figure 11. The controls, with pH of 5.8 and 3.1, germinated very uniformly and germination was nearly complete by 6 days. Low pH appeared to have very little effect. The lower doses of each of ferulic, *p*-coumaric and vanillic acids and coumarin caused some delay in germination (data not shown) but germination was nearly complete by 9 days. The higher doses of each of these compounds strongly inhibited germination. Differences among the toxicities of ferulic, *p*-coumaric and vanillic acids were slight. Coumarin was much more toxic than any of the phenolic acids. This pattern is generally similar to that shown by flax and other seedlings. Inhibition of germination in tobacco was accompanied by severe reduction in seedling size, but this reduction was not measured.

Table 12 shows the effects of ferulic acid on tobacco seedlings. Ferulic acid produced a similiar pattern to that seen in other species: reduction in root length and shoot and root dry weight. Shoot height, although clearly reduced, was not measured.

These results suggested that the overall mechanism of response to these compounds was in the same range as that of other crops examined.

3.1.6 Interaction between phenolic acids in flax and wheat

The effects of combinations of phenolic acids on flax and wheat seedlings were determined. Experimental methods were like those detailed above, except for the fact of mixture, and are listed in Table 4. Results appear in Tables 13 and 14 and Figure 13 . The mixtures were compared to the averaged pure compounds by analysis of variance (ANOVA). In flax, no measurements showed significant departures from additivity at the 0.05 level of probability. In wheat, 7 of 8 measurements showed no significant departures from additivity. In this case, the effect of the mixture of *p*-coumaric acid and ferulic acid on root length was smaller than expected.

The majority of the interactions are additive. This may allow the calculation of

Table 10. Effects of 2x10-3 M p-coumaric acid, 5x10-4 M scopoletin, or 0.95 wild oat extract on barley at 10 days.

Treatment	Variety	Shoot ht., mm		Root length, mm		Shoot dry wt., mg	
control	Conquest	176	100%	132	100%	21.6	100%
	Elrose	136	100%	121	100%	19.1	100%
	Galt	159	100%	137	100%	18.3	100%
	Johnston	171	100%	119	100%	14.8	100%
p-coumaric acid	Conquest	158	90%	82 a	62%	19	88%
	Elrose	130	96%	107 b	89%	17.7	93%
	Galt	136	85%	91 a	66%	16.2	89%
	Johnston	156	91%	83 a	70%	13.2	89%
	Average		91%		72%		90%
	ANOVA interaction	n.s.		**		n.a.	
scopoletin	Conquest	176 a	100%	64 a	48%	22.7	105%
	Elrose	143 a	105%	69 ab	57%	18.3	96%
	Galt	150 a	94%	73 ab	53%	18.3	100%
	Johnston	175 a	102%	72 b	60%	15.7	106%
	Average		100%		55%		102%
	ANOVA interaction	**		*		n.a.	
wild oat extract	Conquest	167	95%	82 a	62%	19.8	92%
	Elrose	137	100%	117 b	97%	20.2	106%
	Galt	150	95%	125 b	91%	18.3	100%
	Johnston	175	102%	109 b	91%	16.8	114%
	Average		98%		85%		103%
	ANOVA interaction	n.s.		**		n.a.	

Responses which are not significantly different at the 0.05 level by Newman-Keul's test are indicated by the same letter, where applicable.

* indicates significant treatment x variety interaction at the 0.05 level; **, at the 0.01 level.

n.s. indicates not significant; n.a., not applicable.

Table 11. Effects of weed extracts on barley cv. Leduc at 10 days.

Expt. #	Treatment	Germination	Shoot length			Root length			Shoot dry wt.		Root dry wt.	
		%	mm	s.e.	% control	mm	s.e.	% control	mg	% control	mg	% control
220	Control	100	149.9	7.4	100	134.4	4.5	100	19.6	100	18.6	100
	stinkweed	100	152.4	7.3	102	100.2	4.3	75	23.9	122	22.3	120
	Canada thistle	100	150.4	9.4	100	117.3	5.0	87	21.6	110	20.7	111
	wild oats	100	149.2	4.4	100	113.3	3.5	86	20.0	104	23.2	125

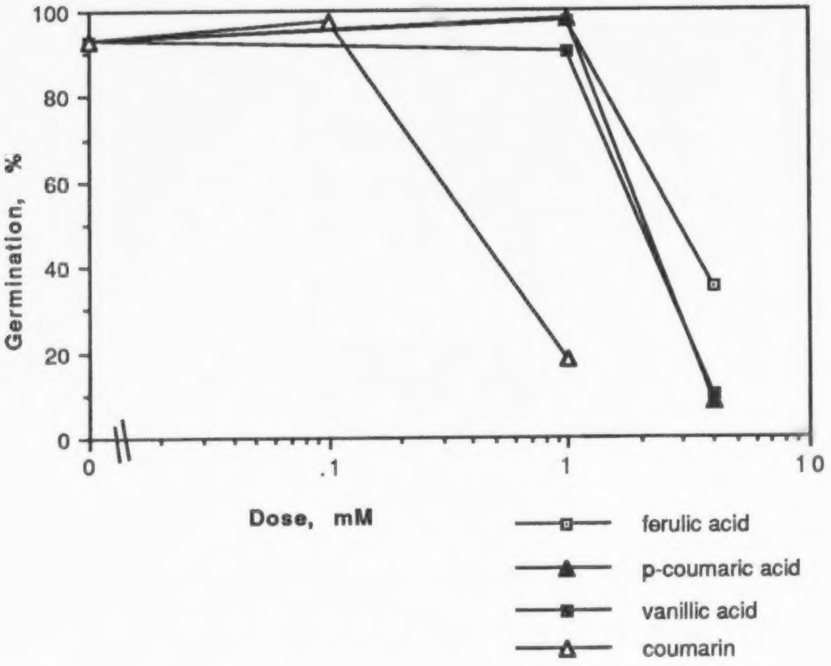


Figure 11. Effects of phenolic compounds on tobacco germination on agar at 13 days.

Table 12. Effects of ferulic acid on vermiculite-grown tobacco seedlings at 23 days.

Treatment	% germination	Root length mm	s.e.	% control	Shoot dry wt. mg	% control	Root dry wt. mg	% control
Control	94	38.3	1.2	100	1.03	100	0.38	100
ferulic acid								
0.1 mM	80	37.2	1.4	98	1.38	133	0.49	129
1 mM	67	31.4	2.0	82	0.56	54	0.25	65
4 mM	73	14.0	0.8	37	0.18	17	n.d.	n.d.

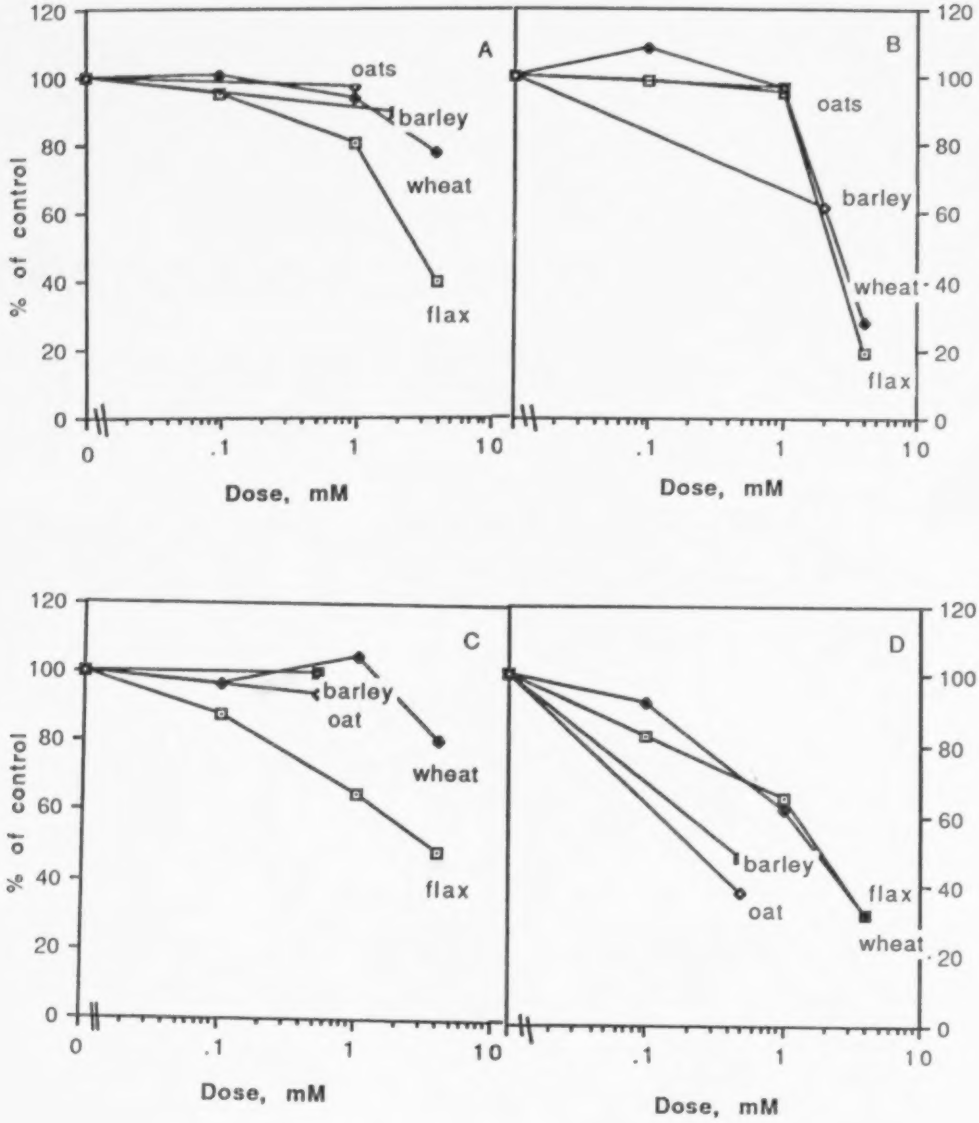


Figure 12. Effects of *p*-coumaric acid and scopoletin on shoot and root length of flax, wheat, oat and barley seedlings.

A, *p*-coumarate on shoot length;
B, *p*-coumarate on root length;
C, scopoletin on shoot length;
D, scopoletin on root length.

Table 13. Additivity between phenolic acids in flax, with unadjusted pH. Expressed as % of control.

	Shoot		Root	
	% of control	Significance	% of control	Significance
control	100		100	
ferulic 4 mM	39.6		30.7	
p-coumaric 4 mM	36.9		19.0	
p-hydroxybenzoic 4 mM	43.7		48.1	
vanillic 4 mM	46.2		28.7	
fer. + pca. 2 mM each	42.1	n.s.	34.1	n.s.
fer. + van. 2 mM each	41.8	n.s.	27.5	n.s.
pca. + van. 2 mM each	42.4	n.s.	26.7	n.s.
all four 1 mM each	43.1	n.s.	29.8	n.s.

* indicates significant departure from additivity at $p<0.05$; **, at $p<0.01$.
n.s. indicates not significant.
ANOVA used to compare results from mixing phenolic acids with average of same phenolics used singly, at same total concentration.

Table 14. Additivity between phenolic acids in wheat, with unadjusted pH. Expressed as % of control.

	Shoot		Root	
	% of control	Significance	% of control	Significance
control	100		100	
ferulic 4 mM	81.6		42.7	
p-coumaric 4 mM	72.5		27.8	
p-hydroxybenzoic 4 mM	95.0		92.8	
vanillic 4 mM	87.4		65.6	
fer. + pca. 2 mM each	82.5	n.s.	47.2	*
fer. + van. 2 mM each	87.9	n.s.	56.0	n.s.
pca. + van. 2 mM each	78.7	n.s.	48.4	n.s.
all four 1 mM each	95.2	n.s.	61.6	n.s.

* indicates significant departure from additivity at $p<0.05$; **, at $p<0.01$.
n.s. indicates not significant.
ANOVA used to compare results from mixing phenolic acids with average of same phenolics used singly, at same total concentration.

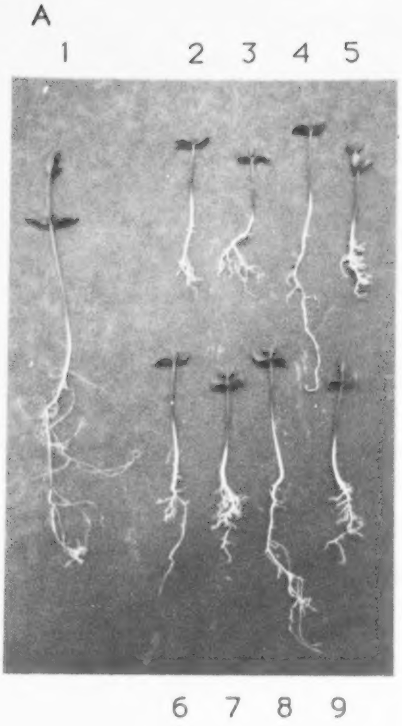
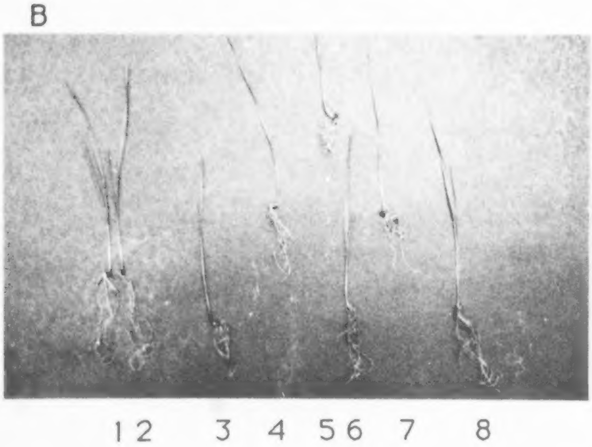


Figure 13. Effects of mixed phenolic acids on flax and wheat seedlings. Each treated seedlings was exposed to 4 mM total phenolic acid.

A, flax. Approximately 0.3X.
1, control;
2, ferulate;
3, *p*-coumarate;
4, *p*-hydroxybenzoate;
5, vanillate;
6, ferulate and *p*-coumarate;
7, ferulate and vanillate;
8, *p*-coumarate and vanillate;
9, *p*-hydroxybenzoate and vanillate.

B, wheat. Approximately 0.2X.
1, control;
2, pH control;
3, *p*-coumarate;
4, ferulate and *p*-coumarate; 5, ferulate; 6,ferulate and vanillate; 7, *p*-coumarate and vanillate; 8, vanillate.



effects of mixtures such as are found in nature, from known toxicity of a single phenolic acid on a particular plant species, and the acid's relative potency. If many species respond similarly, reasonably accurate indices of expected damage could be constructed. Other classes of allelochemicals might also be incorporated into such an index when more information becomes available.

3.1.7 Summary of effects of phenolic acids, coumarins, and weed extracts on crop plants

In sum, the effects of phenolic compounds on the crop species flax, wheat, oats, barley and canola vary from nil, or slight stimulation at low molarity, to severe inhibition of shoot and root extension and germination. Coumarin is clearly the most inhibitory of the chemicals tested, and the only one with a crippling effect on germination. Generally the effects on seedlings correspond well to the hydrophobicity of the compounds, as has been previously noted (Glass and Dunlop, 1974).

Some of the pure phenolic compounds produce "signatures" or very characteristic injuries, exemplified by the necrotic patches seen on flax roots treated with esculetin. It was often possible to pick out which of the randomized flax seedlings had been treated with which compound and at what dose, by a glance at the roots. Such "signatures" were seen on wheat roots also, but less distinctly, and were not very clear or were not examined in the other tested species.

Effects of phenolic acids in combination appear to be additive, that is, equal to the averaged effects of the compounds at the same molarity. Some individual combinations may deviate from this rule, and combinations involving coumarins and other phenolic compounds were not examined. The additivity is likely to mean that phenolic compounds compete for the same sites of activity and that these are not limited in availability.

Overall, flax is the most sensitive to phenolic compounds of the species examined, followed by wheat, barley and oats (Figure 12). The grains respond differently from the dicotyledonous species in that damage is seen more in the root, less in the shoot.

The great variability found among extracts of the same weed species collected at different times and places allows no clearer conclusion to be drawn than that weed extracts may injure crop seedlings and may do so severely. Effects range from nil to extreme. Overall, flax is the most sensitive to weed extracts of the species studied, followed by wheat and barley. Wheat and barley shoots were particularly little damaged, the exception being one lot of Canada thistle on wheat; most damage was confined to the root.

When these results are compared to the literature on yield loss of these crops to weeds, a correspondence appears between response to wild oats in the field (O'Donovan and Sharma, 1983), weed extracts, and phenolic compounds. Flax is most injured in all three cases, followed by wheat, then barley. This correspondence invites speculation about the contribution of allelopathic effects to yield loss. If such a correspondence is confirmed, it would indicate that responses to allelochemicals produced by weeds constitute a major component of these crops' yield losses to weeds.

This correspondence could profitably be studied in more detail, either by correlating crop yield losses to weeds with crop response to weed extracts, over a large number of crop species, or by examining in detail the kinds and quantities of allelochemicals produced by a particular weed and the yield loss produced by this weed, in one or a few crop species, under uniform conditions. Identified genetic variability within a crop species would be valuable in that the variability could be traced through response to weeds, response to weed extracts, and response to allelochemicals produced by the weed. It would be of particular value to have these factors examined together, because of the large variabilities associated with such key factors as weed toxicity.

Virtually no attention has been paid to the genetics involved in any aspect of allelopathy, in either crops or weeds. These limited results indicate that such a project would be valuable in defining more closely the contribution of allelopathy to crop yield loss.

3.2 Intraspecific variation in response to allelochemicals and weed extracts

Intraspecific variation in response to allelochemicals had not been shown prior to the work of A. Kilvert on alfalfa (A. Kilvert, M.Sc. thesis, University of Alberta, 1991). As noted in Section 1.7, however, variation in response to weeds and crop residues has been documented. Variation in response to allelochemicals would establish the significance of allelopathy, in that, where measureable differences exist in a phenomenon, the phenomenon must exist. It would also suggest the possibility of breeding for tolerance of allelochemicals and thence, possibly, for tolerance of weeds. This could be done by testing the tolerance of seedlings to allelochemicals under uniform conditions. Presumptions involved in this chain of reasoning are:

1. that variation in tolerance is genetic in origin and heritable;
2. that allelopathy is a significant component of interference in the field;
3. that degree of tolerance of seedlings is important and/or correlated highly with degree of tolerance of the plant throughout its life cycle; and
4. that effects of weed exudates are accurately and additively represented by pure allelochemicals.

Of these,

1. the likelihood of the existence of genetic variation seemed good, since few traits are not variable to some extent;
2. allelopathy appeared to be a significant component of interference by weeds (39%) in one of the few situations in which it has been distinguished (Bell and Koeppe 1972);
3. since growth is continuous in most agricultural crops, it seemed likely that seedling tolerance and adult tolerance should correlate well, but it should also be noted that the period of seedling establishment is critical for degree of injury by weeds (Sen, 1988); and
4. some data, including those on flax and wheat (Section 3.1.6), indicate additivity at least between allelochemicals of the same chemical classes.

It therefore seemed worth while to determine whether such variation existed in flax and barley. Four cultivars of flax (cv. McGregor, Raja, Indian Type 8 and Abyssinian Brown) and two related species, *Linum halogynum* and *L. pallescens*, were examined in the presence of *p*-coumaric acid. Four cultivars of barley (Conquest, Elrose, Galt and Johnston) were examined in the presence of *p*-coumaric acid, scopoletin and wild oat extract.

Flax results appear in Table 15 and Figures 14 and 15. Differences in response among varieties is indicated by the treatment x variety (=interaction) component of ANOVA. For flax, ANOVA showed that significant differences ($p<0.05$ or 0.01) among varieties exist for shoot height and root length at 3 weeks and shoot height at 6 and 9 weeks. Further analysis by Newman-Keuls' Multiple Range Test (Zar 1984) showed which pairs of varieties differ significantly (Table 15). Since weights of individual seedlings were not found, no statistical tests could be performed on root and shoot dry weight, although they vary substantially (Table 15). The delay in later growth of some varieties was quite apparent. *P*-coumaric acid-treated Abyssinian Brown appears wilty and lax in addition to being much shorter than treated McGregor (Figure 14), for example. Delay in growth varied from 1 day for McGregor to 8 days for Raja (Figure 15). One day is usually an insignificant delay in agricultural terms; eight days is highly significant in growth of field crops in Canada. At flowering, increase in height stopped and the treated plants caught up in height; final shoot height was unaffected. Two

Table 15. Effects of 10-3 M p-coumaric acid on flax at 3, 6, 9, and 19 weeks.

		3 weeks								6 weeks		9 weeks		19 weeks			
Variety		Shoot ht., mm		Root length, mm		Shoot dry wt, mg		Root dry wt., mg		Shoot ht., mm		Shoot ht., mm		Shoot ht., mm		Top dry wt., mg	
Raja	un.	85.4		187		20		22		210		376		499		467	
	tr.	67.3 a	79%	148 ab	79%	11	55%	15	68%	148 a	71%	302 a	80%	500	100%	382	82%
McGregor	un.	90.3		141		19		15		159		261		658		863	
	tr.	79.6 b	88%	126 a	89%	12	63%	10	67%	139 b	87%	264 c	101%	687	104%	704	82%
Indian Type 8	un.	93.4		142		20		21		204		345		688		802	
	tr.	72.8 a	78%	129 a	91%	10	50%	12	57%	156 ab	76%	295 ab	86%	669	97%	671	84%
Abyssinian Brown	un.	81.2		150		14		13		116		203		416		458	
	tr.	61.4 c	73%	99 b	66%	6	43%	5	38%	91 ab	79%	170 ab	83%	428	103%	369	81%
L. halogynum	un.	87.8		159		17		16		160		297		543		534	
	tr.	69.6 a	79%	133 a	84%	9	53%	10	63%	127 ab	79%	248 ab	83%	522	96%	501	94%
L. pallescens	un.	99.4		147		20		18		185		284		600		724	
	tr.	85.1 b	86%	131 a	89%	13	65%	14	78%	153 ab	83%	266 bc	94%	564	94%	634	88%
Average			81%		83%		54%		63%		79%		88%		99%		85%
Interaction component from ANOVA		*		* *		n.a.		n.a.		* *		* *		n.d.		n.a.	

At 3 weeks, seedlings less than 20 mm tall are not considered.

Responses which are not significantly different at the 0.05 level by Newman-Keul's test are indicated by the same letter, where applicable.

* indicates significant treatment x variety interaction at the 0.05 level; **, at the 0.01 level.

n.a. indicates not applicable; n.d., not done; un., untreated; tr., treated.

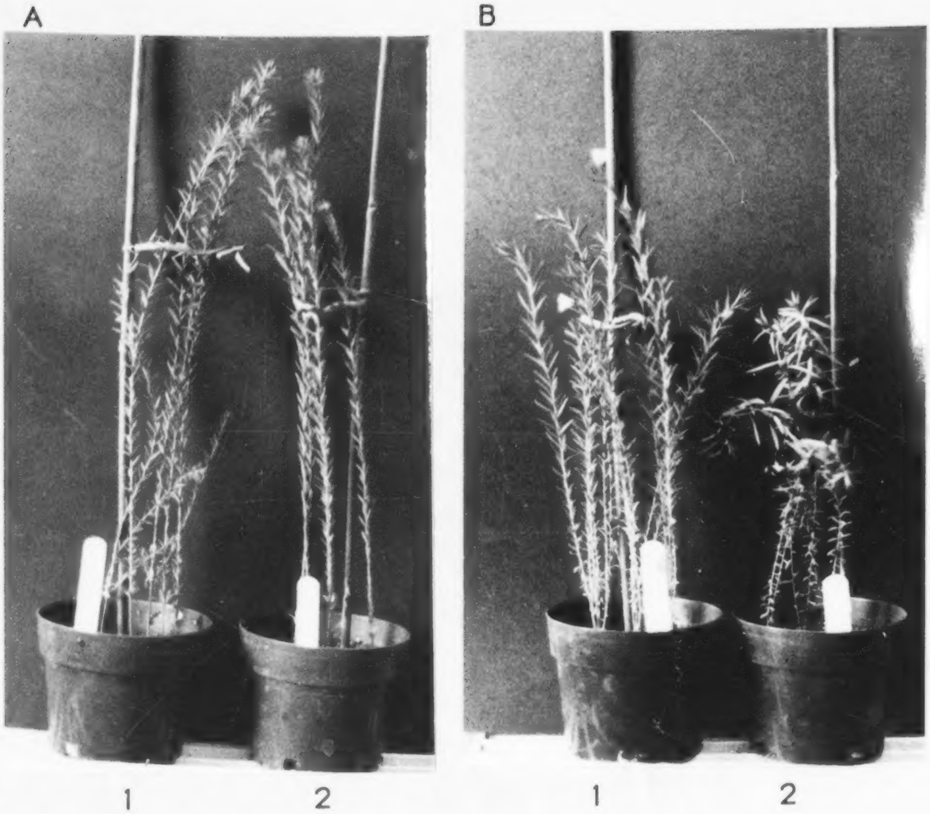


Figure 14. Effects of *p*-coumaric acid on flax cv. McGregor (A) and Abyssinian Brown (B) grown in soil. 1, untreated; 2, treated. Approximately 0.2X.

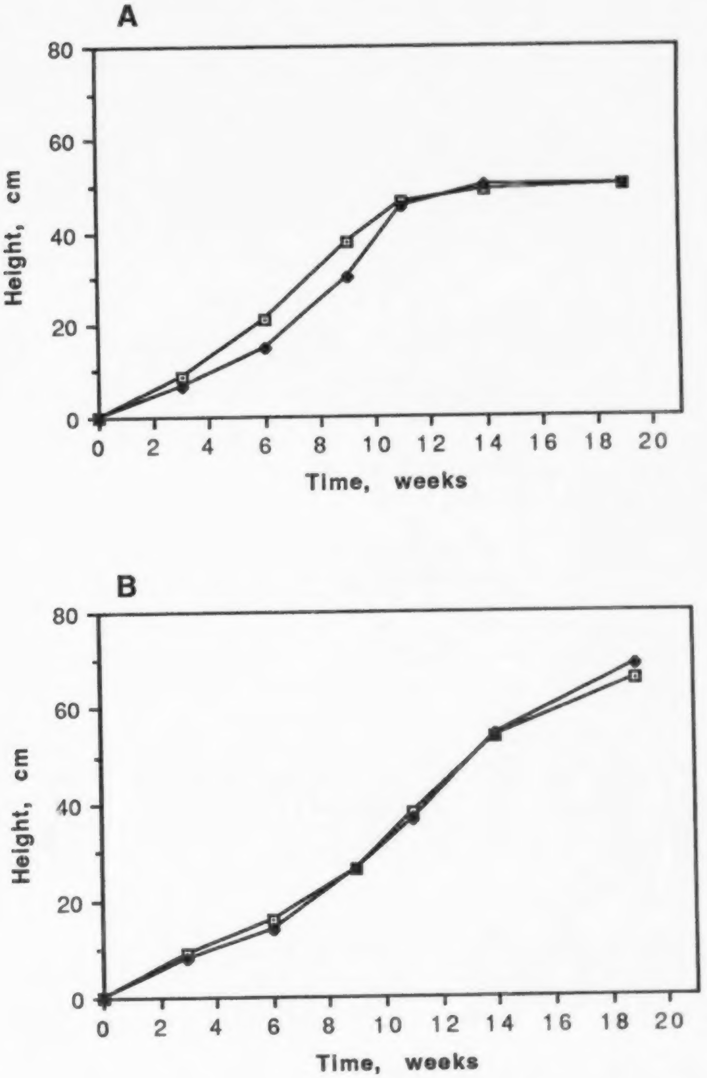


Figure 15. Effects of *p*-coumaric acid on flax varieties Raja (A) and McGregor (B) grown in soil. Square, untreated; diamond, treated.

of the six varieties did not set seed well in the growth chamber so yield data are omitted.

Overall rankings from best to worst would probably run: *L. pallescens*, McGregor, *L. halogynum*, Indian Type 8, Raja, Abyssinian Brown. However, accurate ranking would require careful examination of the contribution of each measured factor to final yield and is outside the purview of this thesis.

Allelochemical dosages used were high but not unrealistically so. Pots of flax received in total the equivalent of 100 kg/ha of *p*-coumaric acid, on a surface area basis; sorghum residue incorporated into soil releases this amount of *p*-coumaric acid (Guenzi and McCalla, 1966).

Barley shoot height and weight were little affected by any treatment, but root length was severely reduced. ANOVA showed significant interaction between variety and all three treatments (*p*-coumaric acid, scopoletin, and wild oat extract) for root length (Table 10). Elrose appeared most tolerant overall, followed by Johnston, Galt and Conquest.

Significant variation in response to allelopathic chemicals appears to exist in flax and barley. While it is possible that this variation in response to allelochemicals is caused by extraneous factors such as seed lot, this is unlikely. Seed of all varieties of barley was from a single source, and five of the six flax varieties from another. Conditions were designed to be as uniform as possible in parameters other than the presence or absence of allelochemicals. The most reasonable explanation seems to be that this variation in response represents genetic variation. If so, this genetic component should be manipulable by standard plant breeding techniques. Testing seedlings by methods similar to those used here should allow identification and selection of more tolerant breeding material for use in cultivar development.

An alternative approach would be to grow cultivars in weedy plots for assessment. Plant breeders traditionally have used perfectly weed-free plots when evaluating new cultivars. Uniformly weedy and non-weedy plots could be used to test yield loss under weedy conditions. For example, a mixture of seeds of important annual weed species could be cross-drilled with varieties being tested. The relative performance of a cultivar with and without weeds could then be accurately assessed. This approach does not distinguish between allelopathic and competitive components of interference, but has the advantages of being simple and clearly related to performance in the field. An approach of this type appears to have been taken by Forcella (1990) in evaluating a soybean cultivar selected for high leaf area expansion rate; it yielded 29% better than one of the parents in the presence of weeds and reduced the growth of weeds by 38%. Identification of more weed-tolerant cultivars could be a valuable adjunct to or in some cases replacement of other methods of weed control.

The work on intraspecific variation in flax and tobacco has recently been published (Ray and Hastings, 1992).

3.3 Transformation of P450 genes into tobacco and flax

Two P450 genes, *Cyp*1A1 and *Cyp*2A5, were chosen for transformation into plants on the basis of potential for detoxification of allelochemicals. They were subcloned into the *Agrobacterium tumefaciens* plasmid pPCV701 (Koncz et al., 1987) with very slight modification. Their structure was confirmed by restriction enzyme digests and Southern blotting (not shown). The recombinant plasmids were conveyed into *Agrobacterium tumefaciens* strain GV3101 by transconjugation via *E. coli* SM10 (Simon et al., 1983) or by freeze-thaw transformation (Burow et al., 1990). This *Agrobacterium* strain contains the helper plasmid pMP90RK which carries necessary virulence genes from the original 200 kb Ti plasmid of *A. tumefaciens* (Koncz et al., 1987). Colonies resistant to both ampicillin and kanamycin were selected. Activity of luciferase was then used to screen the colonies, and the brightest colonies were used to transform tobacco leaf slices (Horsch et al., 1985) or 2-3 day old flax hypocotyl sections (Basiran et al., 1987; Draper et al., 1988). Kanamycin resistant plant material (callus or shoots) was screened for luciferase activity.

In tobacco, transformed cells regenerated into leafy shoots, which were excised and placed on shooting and later rooting medium. Rooted plantlets were gradually moved to soil. In flax, transformation of cells which form callus and shooty material is very common but regeneration from these is not. When transformed shoots were found, they were placed first on shooting and later on rooting medium.

Fertile tobacco plants of normal phenotype were recovered. They were screened for bioluminescence. Southern blots confirmed that the P450 and *lux*F genes were present without obvious rearrangement. Growth of seedling progeny of self-fertilized transgenic plants on kanamycin showed 3:1 kan^r:kan^s segregation in most cases, indicating a single site of integration was most likely. Northern and RNA slot blots showed P450 transcription. The presence of P450 enzyme was assayed by Western blots and protein slot blots, activity by fluorometric assays of crude cell extracts or microsome fractions. Phenotype was examined in seedling offspring of self-fertilized transgenic plants. Seedling growth rates were determined under controlled and uniform conditions, in the presence or absence of phenolic compounds.

In flax, difficulties were met in transferring rooted plantlets to soil and getting them to flower. Limited assays were done on the types of tissue available.

3.3.1 Plant transformation

Tobacco may be the easiest of plants to transform or to regenerate. Tobacco leaf pieces were transformed and readily regenerated shoots and some callus from cut edges. These shoots could be excised at a suitable size and grown into plantlets on selective medium. The plantlets regenerated adventitious roots on hormone-free medium and could be readily transferred to soil. The phenotype of the transgenic plants appeared normal in every macroscopic feature. No histological examination was made.

Flax is easy to transform or to regenerate but it is difficult to regenerate transformed material (Dong and McHughen, 1991; Jordan and McHughen, 1988b). The consensus of opinion appears to be that regenerable cells are few and far from the surface, so that the likelihood of bacteria reaching such cells is low.

Successful flax transformation and regeneration has been reported using three strategies. Transformation of 7-10 day hypocotyl sections peeled of epidermis, followed by direct shoot and later root regeneration (Jordan and McHughen, 1988a, b) has been successful. Shoots form and are selected within several weeks. This method gives rise to a large number of escapes (Jordan and McHughen, 1988b), but has also produced several transgenic derivatives of McGregor flax resistant to the herbicide sulfonurea which are now in field trials (McHughen and Rowland, 1991; McHughen and Holm, 1991). A second strategy is transformation of 1-2 day old hypocotyl sections, followed by callus formation. From these calli arise a few non-transformed shoots, normal in appearance, which if removed may leave a site which then produces small transformed shoots which proliferate and may later be rooted (Basiran et al., 1987; Draper et al., 1988). This method takes several months to produce shoots; however, once formed they will proliferate indefinitely. Excised shoots root at moderately low frequency. Basiran et al. (1987) showed transformation but not seed production from transformants. Transformants from this method can flower (Basiran, 1988). Zhan et al. (1988) attempted a variety of transformation strategies using *A. tumefaciens* and *A. rhizogenes*, another transforming species of bacterium which produces hairy root disease in many dicotyledonous plants. With *A. tumefaciens*, vacuum infiltration or abrasion of 7 day old hypocotyls gave rise to numerous non-transformed but no transformed shoots.

Inoculation of cotyledon sections was also unsuccessful, and addition of sinapic acid had no effect. *A. rhizogenes* was used to successfully transform cotyledon sections, which formed adventitious roots and then shoots in small numbers. About 0.5% of cotyledons eventually produced transformed shoots. The transformants had abnormal morphology including curled leaves, very short internodes, and frequently plagiotropic roots; side shoots sometimes recovered normal morphology on subculture. Seed production was not reported.

The flax sections transformed with P450 genes at first grew callus at the cut ends. Much of this callus died under selection; some remained. After several transfers, green transformed callus could be clearly distinguished from dying nontransformed callus. From yellow or green callus occasionally appeared shoots of normal form and pale in colour. These shoots were found to be non-luminescent, and they did not survive if excised and placed directly in medium containing kanamycin; they were, therefore, escapes. Around the sites on the calli where these shoots were excised, small dark green shoots of different form then appeared. They were very dark green, with small rounded glossy leaves and short internodes (Figure 16A). On excision these propagated freely in the presence of kanamycin. Some retained much the same form; others on subculture regained the opacity, longer internodes, and lighter colour typical of normal flax shoots. A few shoots developed monstrous forms, such as wide twisted flattened stems or staghorn forms topped with tightly-packed small leaves. The dark green shoots which developed later were transgenic as shown by luminometry and Southern and Northern blotting.

Shoots of normal phenotype, mostly without callus, were excised and placed on MS or MS with 0.05 µg/ml kinetin or 0.1-0.5 µg/ml IAA for root regeneration. In many cases they produced bulbous or disorganised callus; in some they vigourously formed adventitious roots (Figure 16B, C). Such rooted plantlets appeared excellent candidates for growth into fertile plants.

For growth into flowering plants, plantlets were placed on a variety of media. Some were transferred to soil or sterile or nonsterile vermiculite wetted with 0.5X MS salts, protected from dessication with clear plastic. Other plantlets were transferred to large graduated cylinders containing 300 ml MS with 0.05 mg/ml kinetin; others, to hydroponic culture with 0.5X MS salts. All were grown in moderate light.

Plantlets transferred to vermiculite or soil invariably died within several days, whether sterile or nonsterile, with symptoms suggesting sometimes dessication and other times root rot. Those transferred to MS in graduated cylinders grew very well at first. Some developed extensive root systems and several attained a height of 20-30 cm with apparently normal morphology. They then began to die from the top, the upper leaves wilting, then the whole plant dying. Well rooted plantlets transferred to a hydroponic system with 0.5X MS salts, completely protected with clear plastic, also died immediately of apparent dessication. Flax grown from seed thrived under these circumstances.

To date (2 years after initial plant transformation), of about 80 rooted plantlets, none have flowered or grown in vermiculite or soil. Several potential reasons for their poor success exist. The trangenotes appear very susceptible to wilting. They may have reduced wax on leaves or inefficient roots or roots which are not well connected to the vascular system. In cases where callus is present at the shoot-root junction this is likely. However, some of the plantlets had numerous roots and complete absence of callus. The transgenotes also appear very susceptible to contamination. It is my impression that fungal contaminants grow extremely fast in these cultures. If so, this might indicate that the plantlets have grown so long under sterile conditions that the normal mechanisms of defense have been down-regulated or lost through mutation. It is also possible that the transgenotes were long enough in culture that they suffered deleterious mutations in, for example, normal patterns of phytohormone production or response, or photosynthetic apparatus.

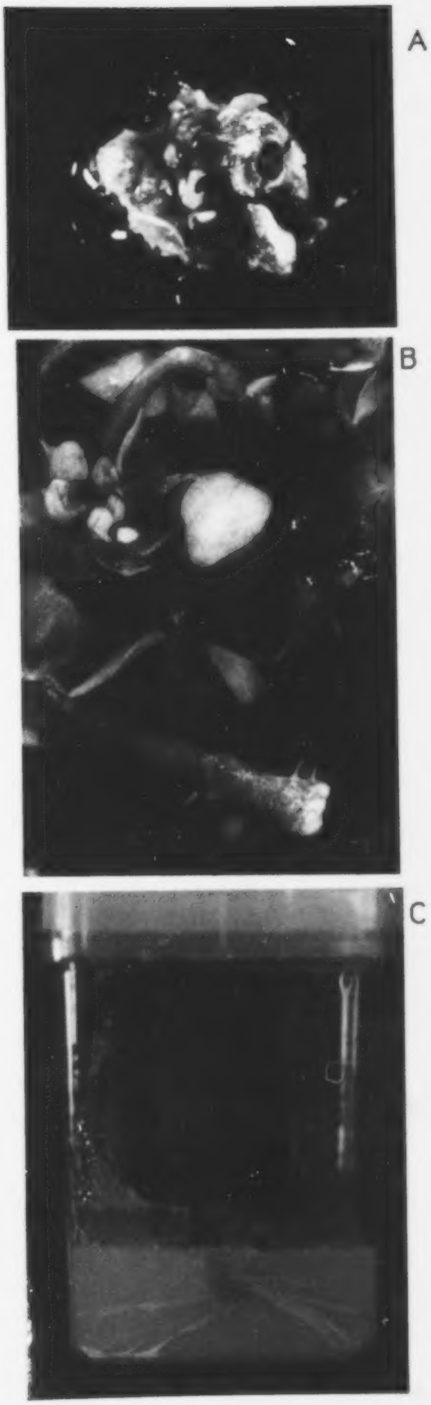


Figure 16. Regeneration of transgenic flax from hypocotyl sections.

A, dark green late shoots arising from pale callus. Approximately 4X.

B, shoots generating adventitious roots. Approximately 4X.

C, shoots with root development. Approximately 0.7X.

Nontransformed flax is easily induced to form shoots and roots from callus or cultured hypocotyl and can readily be brought to flower.

Draper et al. (1988) and McHughen (personal communication) report similar difficulties in regenerating transformed flax. Extensive experiments might assign a specific reason for the phenomenon. At present it seems most likely that long-term tissue culture has induced a condition of extreme tenderness which makes several of the steps in regeneration unusually difficult.

3.3.2 Luminometry

Luceriferase activity of transgenic tobacco plants was measured. Levels in the young transgenic plants were low, but gradually increased to medium levels (0.3-2 LU/mg tissue in outer edge of lower leaf) as the plants matured. Intensity of expression was markedly increased by setting leaf discs on filter paper wetted with 2,4-D or coumarin overnight, when cells were placed in callus/suspension medium (MS with 0.5 µg/ml 2,4-D) or in MS medium containing coumarin. Coumarin responses are described in Section 3.3.3.5. Luminometry gives highly variable numbers from any type of plant material; this seems to be inherent in the method. Representative data from various transgenic tobacco materials appear in Table 16.

Various flax tissues from regenerating cultures were also luminescent (Table 17). They were maintained on medium with a preponderance of cytokinin rather than auxin, so luciferase activity is expected to be low, although high readings were occasionally obtained (Table 17). Since autotrophic flowering plants have not been obtained, "natural" levels of luminescence are not known.

3.3.3 Southern blots

DNA from luminescent plants was digested with *Eco*RI and examined by Southern blotting. Nearly all luminescent tobacco plants proved to carry the expected *Cyp*1A1 fragment at 3.6 kb or *Cyp*2A5 at 3.5 kb. Typical Southern blots are shown in Figure 17; results are tabulated in Table 16. About 45 tobacco plants carried mouse P450 genes.

In a small number of plants, rearrangement of the transformed DNA seemed likely; larger and smaller bands, in addition to that expected, appeared (Section 3.3.4). The nature of the rearrangements was not investigated, nor was copy number.

Callus and shooty material of flax also proved to carry the expected sequences from P450 genes when DNA was digested with *Eco*RI (Figure 17A, C). No flax with obvious rearrangements was found.

3.3.4 Mendelian transmission of transformed genes to tobacco progeny

Transmission of the transgenic traits was tested in progeny of self-fertilized transgenic tobacco. If the transgenotes arose through insertion of DNA at a single site in the genome, the offspring were expected to segregate in a 3:1 ratio for luminescence, kanamycin resistance, and the presence of the P450 gene.

To test kanamycin resistance, seeds from a number of self-fertilized plants were placed on MS salts with agar containing 0 or 100 µg/ml kanamycin. Under these circumstances all seedlings germinated, but kanamycin sensitive seedlings on kanamycin gradually blanched and never developed true leaves, while kanamycin resistant plants remained green and grew. SR1 (non-transformed) and #5 (transformed with *lux*A and *lux*B) seedlings were used as controls. Among the progeny of some self-fertilized P450-

Table 16. Summary of properties of transgenic tobacco material.

Name*	Material	LU/mg, range	DNA	RNA^			Protein		Enzyme Assay	
			Southern	Slot blot	Northern		Slot blot	Western	Microsome	Crude extract
			P450 DNA	P450 DNA	P450 DNA	luxF DNA				
#5	leaf	0.4-1.9	no							
	callus	2.5-15.2		no	no	yes	no		no	
	suspension	1.6-12.5			no	yes	no	no	no	no
4214	leaf		yes							
	callus	0.8					no		no	
	suspension	0.5-9.1					no		no	
42116	leaf	1.9-2.4	yes							
	callus	0.9-3.2					?			
	suspension	2.6-13.1			yes	yes	no, ?	no	no	no
4811	leaf	0.6-0.7								
	callus	4.1					?		no	
4815	leaf	0.5-2.0	?							
	callus						?			
	suspension	2.0-4.7					?		no	
14611	leaf	0.5-0.6	yes							
	callus	0.2-0.3					?			
	suspension	1.2-5.7					?		no	
1063	leaf	0.3-3.0	yes	yes						
	callus	0.6-2.7		yes	yes		?		no	
	suspension	0.6-7.6		yes	yes		no, ?	no	no	no
1067	leaf	0.8-3.5	yes	yes						
	callus	0.5-11.1		yes			?		no	
	suspension	2.7-8.0								
10628	leaf	0.3-0.8	yes							
	callus	1.3-5.3					?		no	
10632	leaf	0.5-1.4	yes							
	callus	0.3-1.2		yes			?		no	
	suspension	0.2-8.7		yes			?		no	

* Plant names beginning with 4 are transformed with Cyp1A1 in forward orientation; those beginning with 14, with Cyp1A1 in reverse orientation; and those beginning with 10, with Cyp2A5 in forward orientation.

^ probed with DNA as indicated.

'yes' or 'no' indicates presence or absence, respectively, of DNA, RNA, protein or enzyme activity.

Table 17. Summary of properties of transgenic flax material.

Name*	Material	LU/mg, range	DNA	RNA^			Protein	Enzyme Assay
			Southern	Slot blot	Northern		Slot blot	Microsome
			P450 DNA	P450 DNA	P450 DNA	luxF DNA		
421	yellow callus on 2,4-D	0.2		yes				
	shoot on 2,4-D	0.2		yes			?	no
	shooty on kinetin			yes				
146	shooty	2.8						
105	pale callus	0.5						
	green callus on 2,4-D	0.7	yes					
	shooty on IAA	0.4			no	no		
105-2	green callus	0.3-1.8	?		yes	no		
105-10	callus on IAA	0.2-0.3	yes					
106	green callus on BA/NA	7.4						
106-5	callus on BA/NAA	0.4	yes				?	

* Names beginning with 4 are transformed with Cyp1A1 in forward orientation; those beginning with 14, with Cyp1A1 in reverse orientation; and those beginning with 10, with Cyp2A5 in forward orientation.

^ probed with DNA as indicated.

'yes' or 'no' indicates presence or absence, respectively, of DNA, RNA, protein or enzyme activity.

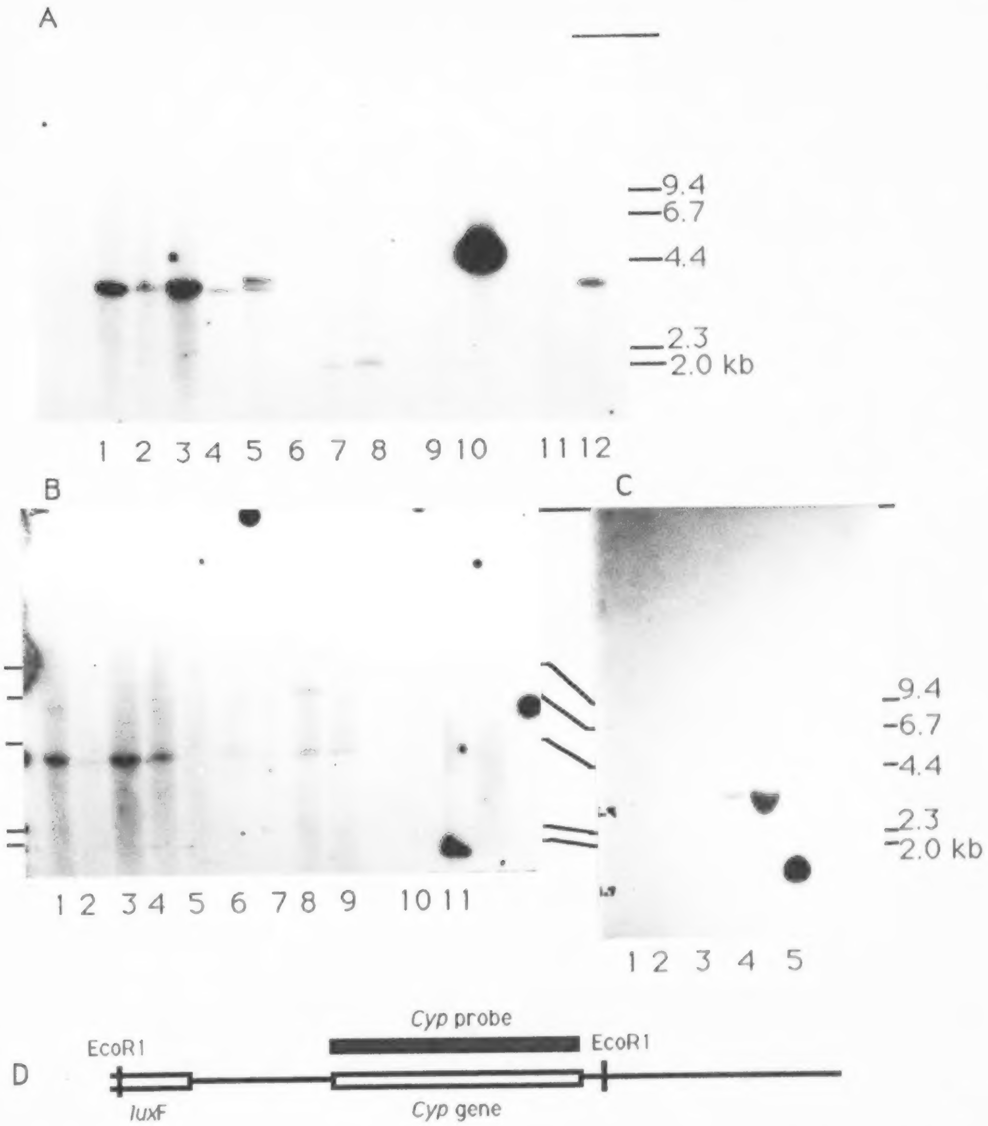

A
—9.4
—6.7
—4.4
—2.3
—2.0 kb
1 2 3 4 5 6 7 8 9 10 11 12
B
1 2 3 4 5 6 7 8 9 10 11
C
-9.4
-6.7
-4.4
-2.3
-2.0 kb
1 2 3 4 5
D
Cyp probe
EcoR1
EcoR1
luxF
Cyp gene

Figure 17. Southern blot of transgenic tobacco and flax. 10µg EcoR1-cut DNA per slot. A, probed with *Cyp*1A1 fragment. **1**, tobacco 4214; **2**, 4216; **3**, 42113; **4**, 42116; **5**, 42127; **6**, 4815; **7**, 14313; **8**, 14611; **9**, SR1; **10**, SR1 mixed with 4.1 kB fragment containing *Cyp*1A1; **11**, McGregor flax; **12**, flax 421.
B, tobacco probed with *Cyp*2A5 fragment. **1**, tobacco 1063; **2**, 1067; **3**, 10611; **4**, 10615; **5**, 10616; **6**, 10625; **7**, 10628; **8**, 10632; **9**, 10635; **10**, SR1; **11**, SR1 mixed with 1.8 kb fragment containing *Cyp*2A5.
C, flax probed with *Cyp*2A5 fragment. **1**, McGregor flax; **2**, 105-2; **3**, 105-5; **4**, 105-10; **5**, McGregor flax mixed with 1.8 kb fragment containing *Cyp*2A5.
D, sketch showing fragment detected in plant.

transformed plants, the following results were obtained:

plant number	green	white	total	$\chi 2$ for 3:1 fit	significant deviation from fit at0.05 level of probability
SR1	0	10	10		
# 5	12	0	12		
4811	24	9	33	0.09	no
4815	27	7	34	0.35	no
4214	35	0	35	11.67	yes
14313	11	3	14	0.09	no
10615	25	4	29	1.94	no
10632	27	9	36	0	no

(Note that plant numbers beginning with 4 are transformed with *Cyp*1A1 in correct orientation, those beginning with 14 are transformed with *Cyp*1A1 in reverse orientation, and those beginning with 10 are transformed with *Cyp*2A5 in correct orientation.) These results indicate that kanamycin resistance was transmitted through meiosis, that segregation took place in a Mendelian fashion, and that in most cases integration occurred at a single chromosomal site in the parent plants. Plant 4214 may be homozygous as occasionally happens with transgenotes.

Smaller numbers of progeny were tested for presence of *Cyp*1A1 by Southern blotting. DNA from progeny of three plants was digested with *Eco*RI and examined by Southern blotting. The results appear in Figure 18. These progeny were also grown in culture with and without 100 µg/ml kanamycin.

None of the progeny of plant 1069 inherited the gene. They were, however, kanamycin resistant. Examination of the parent plant showed it to be negative by Southern blotting and of doubtful luminescence, but resistant to kanamycin. It is possible that rearrangement occurred so that the NPTII but not the *lux*F or the P450 genes were integrated, or that the plant material mutated to kanamycin resistance.

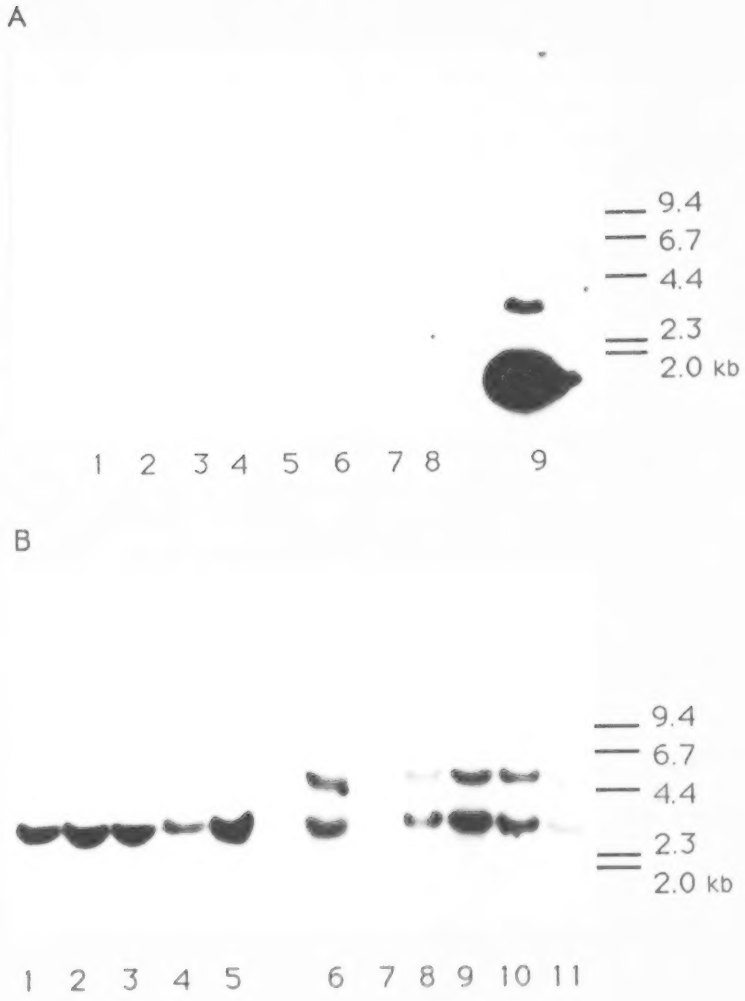
Among the progeny of plant 10616, rearrangement is evident. A larger fragment (about 6.0 kb) appears in addition to that expected. Since these plants are luminescent and kanamycin resistant and do contain the expected fragment, it is probable that the parent contained one normal and one rearranged copy of the T-DNA. The parent contains the same rearrangement. One of the six seedlings tested has not inherited the transgenic suite of characteristics; it lacks *Cyp*1A1 and is non-luminescent and sensitive to kanamycin.

Among the progeny of plant 10613 the fragment is that expected.

The majority of cases are consistent with Mendelian transmission of a gene or genes integrated at a single chromosomal locus.

3.3.5 Expression of transgenes

Expression of the P450 genes in tobacco was assayed by Northern and RNA slot blots, enzyme assays of crude cell and microsome fractions, Western and protein slot blots, and growth trials of whole transgenic seedlings. More limited assays (Northern and RNA slot blots and protein slot blots) were performed on transgenic flax material.


A
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1 2 3 4 5 6 7 8 9
B
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2.3
2.0 kb
1 2 3 4 5 6 7 8 9 10 11

Figure 18. Southern blots of progeny of self-fertilized tobacco transformed with p10. 10 μg DNA was digested with EcoR1 and probed with *Cyp*2A5 fragment.
A, **1-8**, individual progeny of plant 1069; **9**, SR1 DNA mixed with plasmid containing *Cyp*2A5 1.8 kb EcoR1-EcoR1 fragment.
B, **1-5**, individual progeny of plant 10613; **6-11**, individual progeny of plant 10616.

3.3.5.1 Northern blots

RNA was isolated from transformed tobacco suspended cells and flax callus and shooty material. Northern blots were prepared and probed with P450 and luciferase DNA fragments (Figure 19). Messenger RNA from *Cyp*1A1 RNA is about 2.1 kb, appropriate for a gene 1.8 kb long plus a polyadenylate tail, while *Cyp*2A5 RNA is about 2.0 kb from a gene of 1.7 kb. *Lux*A and *lux*B are almost identical in size, giving a double band of 1.5-1.6 kb from genes of 1.1 and 1.0 kb, in the control plant #5, while *lux*F in the *Cyp*-transformed plants gives a band of 2.8 kb from a gene of 2.2 kb. These indicate rather long polyadenylate tails. The amount of luciferase mRNA present correlates well with light emission measurements made on the same material at about the same time, and to the amount of P450 mRNA seen.

Flax produces much less P450 mRNA; luciferase activity and *lux*F mRNA levels are also much lower (Figure 19). However, flax RNA was not enriched for poly(A)$^+$ RNA.

RNA slot blots (using total cellular RNA) reveal intermittently high levels of P450 RNA in callus and leaf from other tobacco and flax material also (not shown). These results are summarized in Tables 16 and 17. RNA and luciferase activity levels in leaf are much lower than those attainable in callus. The *mas* promoter is normally weaker in leaves than in callus (Langridge et al., 1989), and the same pattern is seen here. Other uncharacterised conditions must also affect RNA levels. Callus from the same plant, in apparent good health and growing grapidly under uniform conditions, can produce luciferase activity and RNA levels which vary enormously between samples.

The introduced P450 genes, therefore, appear to be transcribed into mRNA of appropriate sizes at significant levels.

3.3.5.2. Western blots

Transgenic plant tissues were examined for P450 protein by Western blot and protein slot blot, using P450 antibodies provided by Dr. D. Pompon and Dr. M. Negishi. Microsome-enriched fractions were prepared, since P450 enzymes are normally located in the smooth endoplasmic reticulum. Crude cell lysate was also tested, in case the proteins were lost from these membranes during preparation.

Western blots were prepared from crude extract and microsome fractions of luminescent 42116 suspended cells, from a culture known to produce *Cyp*1A1 mRNA. Transfer of protein to nitrocellulose was estimated at 70% or more, indicating 0.7-4.0 mg crude protein in each lane. The positive control (S9 fraction) was detected by P4501A1 antibody, but no other sample. Slot blots from these samples were also entirely negative. At least in this culture, it appears either that no protein was produced or that no protein could be isolated, although mRNA was present.

Protein slot blots suggest that P450 protein is made by some cultures, but sufficient background staining to #5 material (a control lacking introduced P450 genes) occurs to make these results inconclusive (not shown). Background recognition or possibly cross recognition of a plant P450 enzyme may be occurring. Such cross recognitions are common among mammalian P450 enzymes (Waxman, 1991).

In at least some cases it is apparent that protein is not isolated at detectable levels, despite the presence of mRNA. Comparison of luciferase activity levels and antigenicity suggest that the amounts of luciferase made by these materials are marginal for detection by antibody (W. Langridge, personal communication). Since the levels of luciferase RNA and P450 RNA are similar (Figure 19), levels of P450 protein are probably also marginal for detection by antibodies. Higashi (1985) and Gabriac et al. (1991) also found low productivity and/or high lability of P450s in plants.

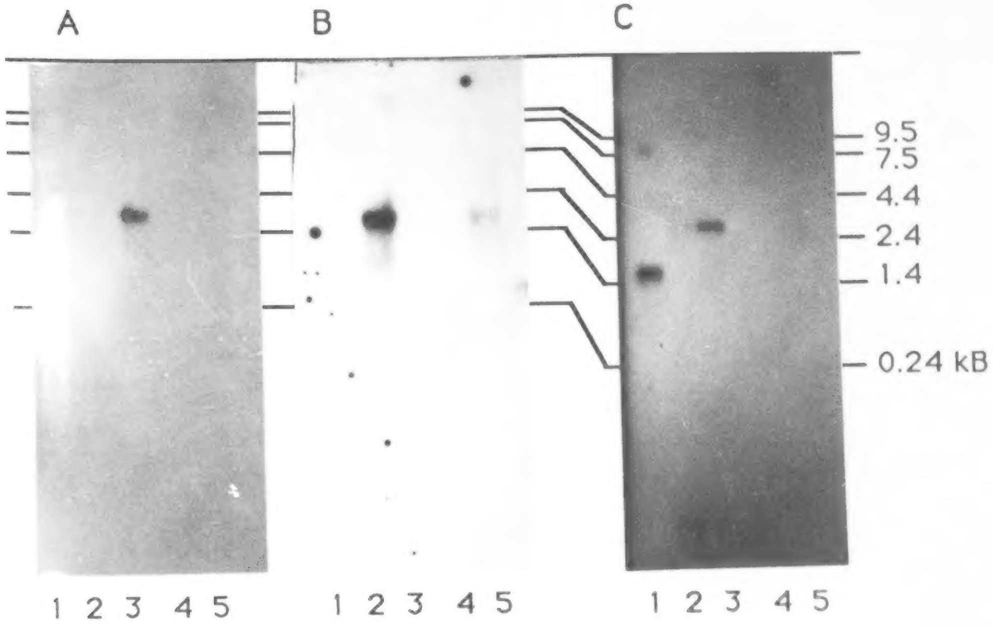


Figure 19. Northern blots of transgenic tobacco and flax.
A, probed with *Cyp*1A1 DNA fragment;
B, probed with *Cyp*2A5 DNA fragment;
C, probed with *lux*F DNA fragment;
1, 7 µg poly(A)+ RNA from #5 tobacco suspension (9.1 LU/mg);
2, 7 µg poly (A)+ RNA from 1063 tobacco suspension (1.6 LU/mg);
3, 7 µg poly (A)+ RNA from 42116 tobacco suspension (3.9 LU/mg);
4, 10 µg whole RNA from 105-2 flax callus (0.73 LU/mg);
5, 10 µg whole RNA from 105 flax shoot (0.42 LU/mg).

3.3.5.3 Enzyme activity

Enzyme activity in crude cell and microsome fractions was examined by fluorometry and UV scanning. Activity of microsome samples and crude cell extracts was assayed directly in a fluorometer cuvette. Under these conditions rat S9 fraction (containing the rat form of P450IA1 enzyme) converted 7-ethoxyresorufin to 7-hydroxyresorufin at a constant rate of 2-3 nmol/mg total protein/second (Figure 20A). If supplied with additional substrate and NADPH, the reaction continued for several minutes. Rat S9 fraction also converted 7-ethoxycoumarin to umbelliferone (not shown).

Plant microsomes and crude cell extracts containing *Cyp*1A1 or *Cyp*2A5 were assayed with 7-ethoxyresorufin as substrate for P450IA1 and with coumarin as substrate for P450IIA5. None of the microsome or crude cell extracts from tobacco or flax transformed with *Cyp*1A1 or *Cyp*2A5 have shown any activity to date, by this assay (Figure 20B is typical). Material from a number of tobacco plants and flax sources has been tested with negative results; they are indicated in Tables 16 and 17. Not all the material had been tested for luciferase activity immediately before extraction, but repeated testing on the bright suspension cultures 42116 and 1063 was negative for P450 enzyme activity while luciferase activity was very high and mRNA was being produced (Table 16). Use of two different isolation buffers and, for P4501A1, two different assays, gave uniformly negative results. Addition of the protease inhibitors leupeptin and pepstatin (5µg/ml) to isolation buffers had no effect. Some assays were also run for 15 minutes to 5 hours, but no product was detected over this time.

Several possible causes of this inactivity may be suggested:

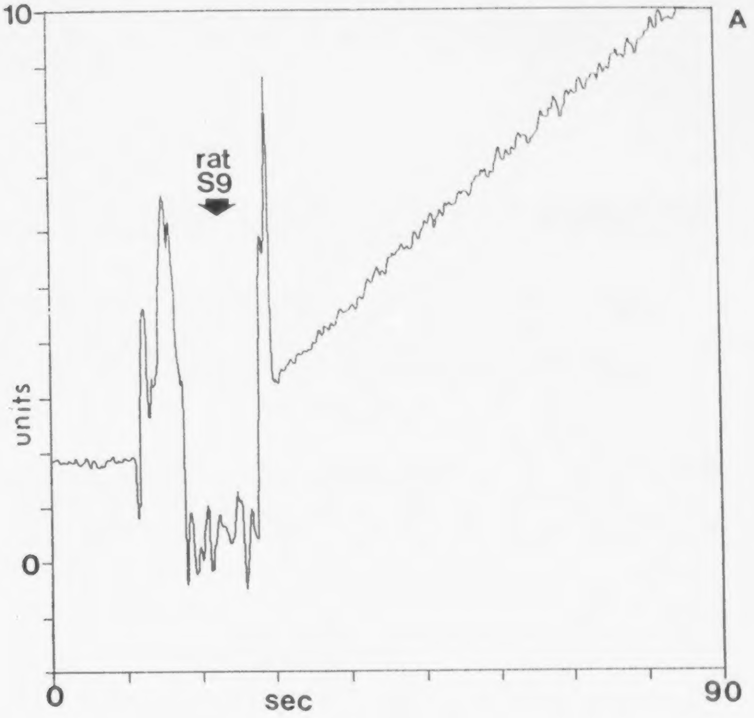
1. insufficient levels of enzyme produced;
2. loss of the enzymes during preparation;
3. inadequate amounts or cooperativity of cofactor, particularly P450 reductase which donates electrons to the enzyme;
5. phosphorylation and consequent inactivation of mouse P450 enzymes in plants; or
6. other inhibiting factors in plant extracts.

Some of these possibilities were examined. To find whether the cell environment was suitable for P450 expression and whether all necessary cofactors were present, I attempted to induce and measure a native P450 enzyme activity. The rationale used was that induction of phenylpropanoid metabolism, which requires activity of the P450 enzyme cinnamate-4-hydroxylase at an early stage, would concommittantly induce the production of enough P450 reductase and other necessary cofactors to support this activity. These cofactors would be expected to be available to foreign P450 enzymes as well.

I attempted to induce the main phenylpropanoid pathway by yeast extract, used as a crude representation of fungal infection, by UV light, which elicits phenylpropanoid production in a large number of plant species, and by manganese, which induced cinnamate-4-hydroxylase in Jerusalem artichoke (*Helianthus tuberosus*) most strongly of several elicitors tested by Reichhart et al. (1980). Cinnamate 4-hydroxylase activity was assayed by UV scanning from 250-330 nm, using the time 0 sample as a control. At 100 minutes a small broad peak appeared centered at 295 nm; this peak was much larger at 300 minutes (Figure 21). It appeared to be *p*-coumaric acid which shows a broad peak centered at 295 nm. Approximately equal amounts of *p*-coumaric acid were produced by all cultures tested. This peak indicates that the plant material, at least as crude cell extract, can support a P450 enzyme activity, the activity is slight, fairly long-lasting and is not induced by yeast extract or UV, at least as used.

If cofactors such as heme or reductase are rate-limiting in cinnamate 4-hydroxylase activity, they are likely to be limiting the introduced P450 enzymes as well. However, the plant extracts as prepared are capable of supporting a P450 enzyme

Figure 20. Enzyme activity of microsome fractions. Fluorometer reading of conversion of 7-ethoxyresorufin to 7-hydroxyresorufin by P450IA1. Excitation 530 nm; emission 586 nm.
A, rat S9 (microsome) fraction (about 0.2 mg protein).
B, typical tobacco microsome fraction (0.1-1.0 mg protein).
Microsome initially present, 7-ethoxyresorufin and NADPH added at first arrow, then more microsome added at second arrow. Order of addition does not affect outcome.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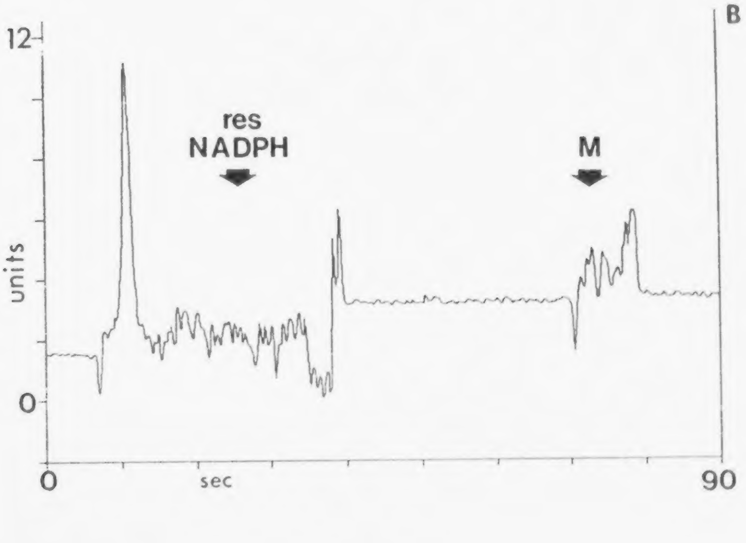
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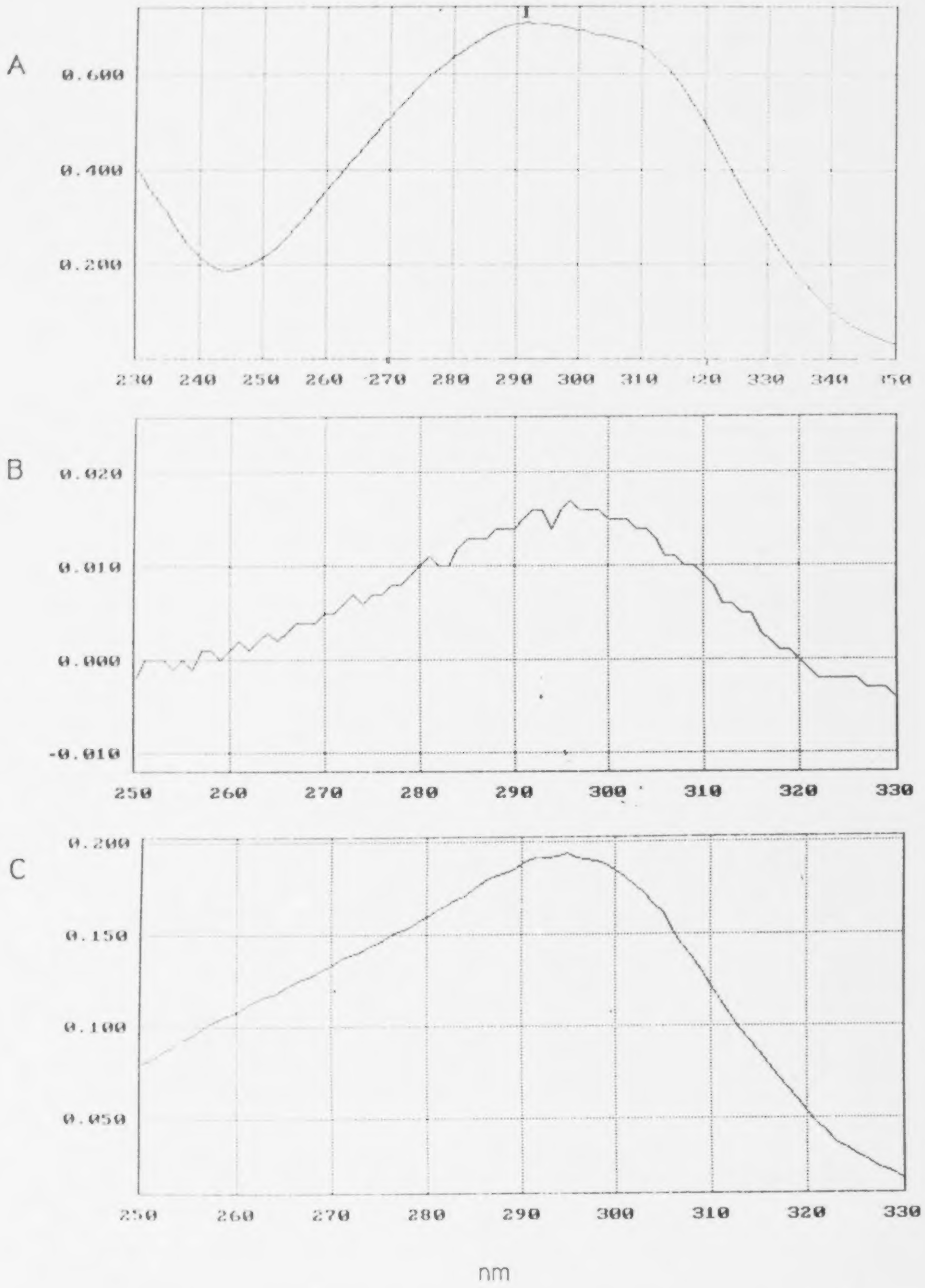

Figure 21. Enzyme activity of cinnamate-4-hydroxylase in crude cell extract of #5 tobacco. UV scan for formation of *p*-coumaric acid (broad peak centered at 295 nm). Reference cell has same amount of reaction mix stopped at time 0.
A, *p*-coumaric acid in buffer.
B, 100 minute incubation.
C, 300 minute incubation. Note differences in scale.

activity.

The possibility of the plant cell producing a P450 inhibitor was examined by adding rat S9 fraction to the plant microsome assay; the S9 fraction reacted at its usual rate (not shown). It is possible that the P450 enzymes made by plant material were permanently inactivated before or during preparation.

Inactivation of P450 enzymes of the same class as P450IIA5 by phosphorylation has been shown in mammalian systems, and the necessary amino acid seqence for recognition by a plant protein kinase (Polya et al., 1989) occurs in P450IIA5. This possibility was investigated by trying to reverse phosphorylation using a phosphatase, and by examining phosphorylation of microsomal proteins *in vitro*.

Enzyme function in crude cell extracts of bright tobacco suspended cells was assayed following pre-incubation with calf alkaline phosphatase. This treatment would be expected to remove phosphate from proteins and therefore to allow P450 enzyme activity if they had been inactivated by phosphorylation. No enzyme activity was observed.

Dr. G. Bagi (Department of Plant Science, University of Alberta) undertook to phosphorylate microsome and crude cell extracts using endogenous enzymes, by incubating these extracts *in vitro* in the presence of ^{32}P-γ-ATP, then separating the proteins by polyacrylamide gel electrophoresis. It appears possible that a band of 48 kD (expected size of P450IIA5 is 49 kD) is phosphorylated more heavily in 1063 cells than in controls, but at both this position and 57 kD (the expected size of P450IA1) other microsomal proteins are also phosphorylated. It is therefore difficult to distinguish the appearance of new bands.

Although the necessary sequences for phosphorylation are present, contraindications are that cAMP-dependent phosphorylation (as seen in the mammalian P450s of this class) appears not to be frequently significant in plants (Trewavas and Gilroy, 1991), that dephosphorylation does not result in activity, and that phosphorylation of proteins of the correct size cannot be shown clearly. These results, therefore, do not indicate that the transformed P450 proteins are present but inhibited by phosphorylation.

The balance of evidence suggests that enzyme levels produced are not sufficient to show activity, but absence of necessary cofactors or presence of inhibiting factors cannot be ruled out.

3.3.5.4 Growth of transgenic seedlings

The growth of whole seedlings with and without *Cyp*2A5 was examined. It seemed possible that biological activity might be present despite the lack of assayable activity in cultured cells, as the *mas* promoter induced high levels of *lux*F activity in root tips (Langridge et al., 1989 and unpublished results).

Tobacco seedlings from self-fertilized transgenic parents segregated in a 3:1 ratio for P450^{+}:P450^{-} (Section 3.3.4). These mixed populations were very suitable for assay of growth rate in the presence or absence of allelochemicals. Coumarin was tested on offspring of self-fertilized tobacco plant 1063. Individual seedlings of unknown luciferase status were grown in the presence or absence of coumarin under otherwise uniform conditions. Their luciferase status was then found and used to indicate their P450 status. The data were sorted accordingly. This type of blind trial should be free of bias in measurement.

Two trials on offspring of the bright plant 1063 were performed. The first showed an advantage in growth for lux^{+} seedlings in the presence of coumarin. However, this result was reversed in the second trial, in which somewhat larger numbers of seedlings were tested. If the two trials are pooled, no difference in growth rate is seen

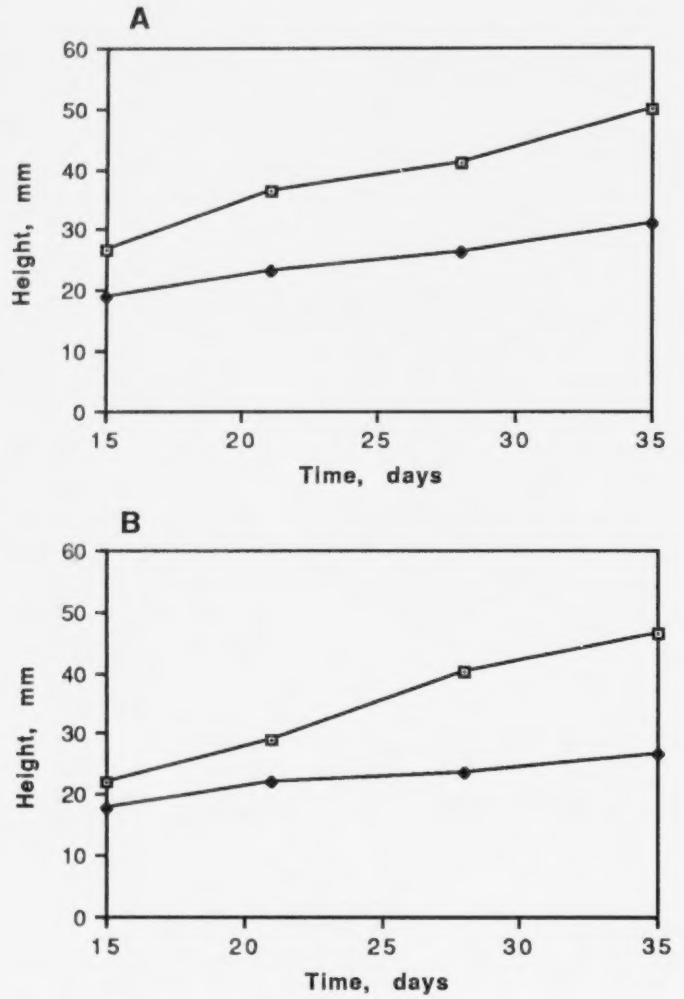

Figure 22. Growth of seedling progeny of plant 1063 from 15 to 35 days after initial treatment with coumarin. Pooled results of 2 trials.

A, non-transformed seedlings.

B, transformed seedlings.

Hollow squares, untreated.

Filled diamonds, treated.

(Figure 22). Lux⁻ seedlings when treated with coumarin grow to 62% of the height of untreated controls; lux⁺ treated seedlings grow to 58% of the height of untreated controls. *Cyp*2A5 does not significantly change the plant's response to coumarin.

3.3.5.5 Effects of coumarin

Coumarin strongly inhibits germination and growth of plants, but may also have the contrary effect of promoting growth by interacting with IAA oxidase. These effects may potentially confuse assays of coumarin effects.

Coumarin is reported to produce auxin-like effects because it competes with IAA for IAA oxidase, thereby inhibiting this enzyme and increasing the available pool of IAA (Einhellig, 1986). Auxin-like effects were observed in transgenic plant #5 and P450-transformed tobacco leaf discs placed on shooting medium (MS with 1 µg/ml BAP and 0.1 µg/ml NAA) containing 0.1-0.4 mM coumarin. Callus production increased dramatically in some cases, while extensive roots formed in others (Figure 23). No difference between the responses of #5 and P450-transformed material was evident. In the absense of coumarin, this medium induces formation of multiple shoots along cut edges of leaf pieces.

The effects on the *mas* promoter of coumarin and 2,4-D were compared, using luminometry. Leaf discs were cut from upper and lower leaves of lux⁺ progeny of self-fertilized plant 1063. Plants were about 12 cm high. Leaf discs were treated for two days with 3 ml 0.5X MS, 0.5X MS with 0.5 mM coumarin (=75µg/ml), or 0.5X MS with 0.1 µg/ml 2,4-D. Luminescence was measured by luminometer. Readings are shown in Table 18. They show that both 2,4-D and coumarin at the doses used act on the *mas* promoter to increase luminescence; coumarin may stimulate it even more than 2,4-D. Levels attained by the lux⁺ progeny of 1063 were uniformly lower than those found in #5 plants. This is probably a result of position of insertion in the genome.

In the case of plants transformed with *Cyp*2A5, this reaction would be expected to amplify the effect on the plant of this gene. That is, conversion of coumarin to umbelliferone should be accelerated and the toxicity of coumarin accordingly reduced, since it stimulates the expression of a protein which destroys it. However, this does not appear to be the case.

Alternatively, if coumarin protects indoleacetic acid (IAA) (Section 1.5), IAA levels will be higher than normal and therefore cell growth and division will increase. Low levels of coumarin also stimulated the growth of suspended cells (not shown). Coumarin could thus increase growth rates above control levels and this effect would be stronger in cultures without active P450IIA5. Thus coumarin hydroxylase activity might obscure evidence of its activity.

3.3.6 Summary of P450 transformation into plants

Of the two P450 genes successfully introduced from mouse into tobacco and flax, both appear to be expressed as judged by presence of mRNA, and it is possible that P450 protein is produced. However, both appear to lack any detectable enzyme function in the plant, and no change in plant or plant cell response to phenolic compounds could be detected. Some potential reasons for this lack of activity include:

1. nontranslation due to cosuppression or mutation of the genes;
2. inadequate preparation methods to show significant amounts of enzyme;
3. lack of suitable cofactors, either because plant phenolic metabolism is very low or because plant reductase cannot interact with the foreign enzyme;
4. inhibition of the introduced enzymes by some factor, possibly phosphorylation; and

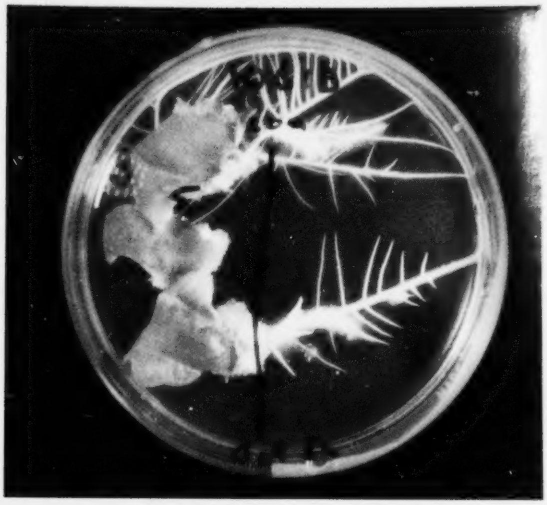

Figure 23. Effects of 0.1 mM coumarin on transgenic tobacco leaf (progeny of 10613) in culture. In the absence of coumarin, shoots and small amounts of callus form. Approximately 0.7X.

Table 18. Effect of coumarin (75 µg/ml) and 2,4-D (0.1 µg/ml) on luminometer readings of leaf discs of lux⁺ progeny of 1063.

Plant number	Leaf location	Readings (LU/ one leaf disc)			
		water	0.5X MS	2,4-D	coumarin
3	lower		7.6	30.6	21.9
	upper		4.5	6.0	11.8
4	lower	2.3	2.5	1.4	
	upper	2.2	0.9	6.6	23.3
6	lower		8.3	15.9	15.2
	upper		26.3	49.6	107.9
7	lower		10.7	15.8	19.4
	upper		5.3	6.7	6.8
10	lower		4.6	8.1	18.0
	upper		44.6	87.6	237
Average	lower		7.8	14.6	15.2
	upper		16.3	31.3	34.7
older 1063 progeny	?		16.9	46.3	43.2
older #5	lower	176	>407	263	>518
	upper	219	>536	457	1086

5. prompt further metabolism of product.

Of these, co-suppression is least likely. This phenomenon has been found to inactivate introduced copies of various genes with homology to those already present. It was discovered as white and pale flowers in a purple-flowering strain of petunia (*Petunia hybrida*) when transformed with an additional copy of the purple pigment gene (Napoli et al., 1990; van der Krol et al., 1990). That is, excess copies of the gene silenced all copies. How such copies are detected and silenced is not known. To date, all cases of co-suppression have involved higher degrees of homology than are likely between plant and mammalian P450 genes (although more than one copy of the new genes may have been introduced), and have apparently involved promoter methylation (Meyer et al., 1992) or transcription followed by lack of processing of primary transcript to mRNA or rapid mRNA destruction, such that mature mRNA could not be detected (de Carvalho et al., 1992). Since *Cyp*1A1 and *Cyp*2A5 are transcribed and the mRNA is polyadenylated, if co-suppression occurs it is of a new kind which interferes with translation.

The mRNA which was produced should have been translated into protein unless the P450 genes were mutated. In fact the *Cyp*2A5 sequence was found to have one base pair change which created a *Pst*I site (Section 2.2.3.1), but no change in amino acid sequence was detected on sequencing through the region. This does not exclude the possibility of other mutations in *Cyp*2A5, nor in *Cyp*1A1. Since several independent transformants were examined for RNA and microsomal assays, for each introduced gene, mutation or somaclonal variation within the plant material is unlikely.

Inadequate sample preparation is possible, particularly since P450s have generally been reported to be produced at low levels and/or to be labile (Gabriac et al., 1991; Juvonen et al., 1988). An extraction method recommended by Zimmerlin et al. (1992) as being better for isolation of plant P450 enzymes gave no evident improvement. Since levels of enzyme detected by Western and protein slot blots were very low or absent, some combination of low production and low recovery of the P450 enzymes is the most likely explanation of their inactivity.

It is possible that mammalian P450 enzymes, if made, might be incompatible with plant reductases. This may have been the case when a plant P450 gene was transformed into yeast and produced protein but no enzyme activity (Vetter et al., 1992). Murakami et al. (1987), examining heterologous expression of a rat P450 in yeast, found that fusion of a reductase gene to the P450 gene (giving a fusion protein) led to a fourfold increase in activity, indicating that reductase was a limiting factor. However, Saito et al. (1991) have successfully expressed a mammalian P450 gene in tobacco, and plant reductase appears to contribute electrons to at least two different plant P450 enzymes (Donaldson and Luster, 1991), and to interact with a mammalian P450 enzyme expressed in *E. coli* membranes (Shet et al., 1993). Since the requirement for the entire phenylpropanoid pathway is reduced to a minimum in these plants and cells, due to the absence of inducing factors, it may be that correspondingly little reductase is made. Activity of the native P450 enzyme, cinnamate-4-hydroxylase, could be detected but with difficulty. It was not induced to higher levels by the treatments attempted. Various other cofactors are also required by the P450 enzymes, some of which might be limiting in plants.

The plant cell environment could be somehow unsuited to the expression of these P450 enzymes. In the whole cell, pH, for example, might be too low. This should not directly affect assays of microsome fractions in buffer. Since the S9 fraction was active in the presence of the plant microsome fraction, no immediately toxic factor was present.

Pyerin and Taniguchi (1989) found, in mammalian liver, a cAMP-dependent protein kinase recognizing the amino acid sequence Arg-Arg-X-Ser or Arg-Arg-X-Thr, which may phosphorylate and thereby inactivate a P450 enzyme of the same family as P450IIA5. The necessary recognition sequence is present in other P450 enzymes of the family (Pyerin et al., 1987), including P450IIA5 (Lindberg et al., 1989). Whether such recognition could occur in a plant background would depend on the plant protein kinase recognition sequences. A current consensus sequence for *calcium*-dependent plant kinases is Arg-X-X-Ser (Polya et al., 1989), a sequence present in both P450IA1 and IIA5. In P450IIA5, Arg-Arg-Phe-Ser is present near residue 128, suggested to be the critical location (Pyerin and Taniguchi, 1989). Phosphorylation around residue 128 may prevent transfer of electrons from reductase (Donaldson and Luster, 1991). In P450IA1, the plant kinase recognition sequence occurs three times, but not in this region of the protein. Phosphorylation of this sequence in plants could potentially be mediated by a calcium-dependent kinase.

Phosphorylation of P450IIA5 appears to be doubtful, since dephosphorylation did not result in any enzyme activity. It is possible that phosphorylation occurred *in vitro* but other proteins of the same sizes as P450 enzymes do not allow this to be shown. It should be noted that in most plants cAMP levels are reportedly too low to support cAMP-dependent kinase activity (Trewavas and Gilroy, 1991).

The possibility that the P450 enzymes are in fact active at low levels but the immediate product of the reaction is promptly further metabolized is slight. When transformed suspension cells are grown in the presence of coumarin or *t*-cinnamic acid, only a small portion of the phenolic compound is withdrawn from the medium, although some glucoside accumulates (not shown). This indicates that the introduced P450 enzymes are not able to draw in large amounts of their substrates.

At least one mammalian P450 gene has been expressed successfully in tobacco. Saito et al. (1991) found mRNA and protein production from a minor P450 form of rabbit (P450IIC4), of unknown function. They did not assay its activity. However, it was in some way sufficient to affect the phenotype of the plant severely. Transformants were unusually slow to arise, quick to flower, grossly deformed, and quick to senesce. This P450 enzyme, therefore, was very active in the whole plant, although it is not known how it acted. The P450IA1 and IIA5 tobacco transformants, on the contrary, were phenotypically normal, vigorous plants.

The activity spectra of mammalian P450 enzymes on allelochemicals have not been of interest to the main body of P450 researchers and are not well understood. Once such information has been generated, some P450 genes may prove useful for reducing allelopathic effects in future. The P450 genes used in this work do not appear to have a useful effect.

4 Summary and further approaches

This thesis establishes that variation within crop species in response to allelochemicals exists and may, therefore, be exploited to breed crops which should be less susceptible to interference by weeds. It does not establish that foreign genes may be usefully introduced to crop species to increase their resistance to allelochemicals. However, it shows several potential lines of research toward this end.

Weeds cannot be controlled by breeding a weaker and less toxic weed. However, they can be indirectly influenced by the breeding of the crop. The effects of the weed on the crop and of the crop on the weed are the same in kind, but the crop species can be more directly manipulated. Plant on plant interactions include toxicity and resistance to toxic compounds. These are allelopathic factors. Other factors include various aspects of competitive abilities and plant architecture. Several genetic approaches may be made to improve the ability of the crop species to thrive despite the presence of the weed. The crop can be made more competitive in terms of efficient use of water, nutrients, and light. Crops varieties with faster early leaf expansion and more closed canopies can suppress weeds by shading. The crop can be made more toxic, or it can be made more resistant to weed toxins.

Of these, only efficiency has received much attention, and then because of the value of these traits in the absence of weeds. A small number of papers has also noted that some varieties suppress weeds better by reason of greater height or faster leaf expansion (Section 1.5). These approaches require no knowledge of allelopathy.

The approaches which involve allelopathy have been essentially unused. Several papers have noted that some crop varieties appear to be more toxic than others, and it is clear that some crops are more toxic than others. Some crops, such as rye and sorghum, lose little yield to weeds, apparently by reason of their toxicity. It may be reasonable to make more susceptible crops similarly toxic. On the other hand, it may be that crop toxicity on the whole has already approached its limit, since crops later in rotation frequently suffer injury from residues of previous crops. For example, following a canola crop it is frequently observed that the succeeding crop comes up more slowly where the canola swath lay. The problem will become more common as low-till and no-till farming spread.

The approach of increasing the resistance of the crop to weed toxins has not previously been studied except by A. Kilvert of this laboratory. It avoids the problems of increased crop toxicity, but its disadvantages are not known. They might include altered response to pathogen infection, or, in crops inclined to become weedy, might make them more capable weeds.

I have examined this approach by examining variation within crop species, thereby establishing the possibility of using artificial selection and by introducing foreign genes of known ability to detoxify particular allelochemicals.

Variation was found to exist within flax and barley and presumably can be exploited either to identify or to select varieties which are more resistant to allelochemicals. Flax and barley differ considerably in sensitivity, but it appears likely that both are capable of improvement. Identification of resistance to reduction of root length and branching might be particularly useful, since the roots are most affected by allelochemicals and this root damage is likely to reduce their competitiveness severely. This may involve altered response to the phytohormone interactions of allelochemicals. The existence of this variability also supports the existence of allelopathy as a real phenomenon.

The introduction of foreign genes to promote the detoxification of allelochemicals was unsuccessful in that no change of phenotype could be detected. After investigation of various other possibilities I selected two mammalian P450 genes with properties that suggested that they could reduce the toxicity of some allelochemicals by hydroxylation. Both were successfully introduced into flax and tobacco and mRNA detected. However, their enzyme function in the plant cell to date is nil. The introduction of the P450 genes has not led to measureable change in the phenotype of whole transgenic tobacco seedlings. Possible explanations for this lack of effect are several. Potential sources of error in the work done are discussed in Section 3.3.6. Further work which might be done includes:

1. examination of the introduced genes by sequencing or by transcription/translation *in vitro*, to be sure that they have not mutated;
2. strong induction of the general phenylpropanoid pathway to provide adequate supplies of reductase, with provision of high levels of iron or heme;
3. use of a promoter which is strongly expressed in the root cells most likely to encounter exogenous allelochemicals;
4. transformation into the plant of mammalian reductase under a strong promoter, or incubation of plant material with mammalian reductase prior to enzyme function assays;
5. Northern blots and enzyme assays from a larger number of plants and tissues, using different methods of P450 enzyme isolation; and
6. determination of kinase activities within plant tissues, if the P450 enzymes are clearly shown to be produced.

4.1 Research in interspecific and intraspecific variation

Regarding natural variation, the major question is: what factors condition a plant's resistance or sensitivity to allelochemicals? Some areas to look at are suggested in Section 1.4; they include glycosylation (Balke et al., 1987), hydroxylation and compartmentalization. Differences are likely to be quantitative and probably small rather than qualitative, and the potential profit of this information is more valuable in weed-susceptible crops such as flax.

In practical terms, it might be useful to assay the performance of new varieties and popular released varieties in the presence as well as the absence of common allelopathic weeds. Such an assay could easily be incorporated into the later plot stages of new variety testing, just as pathogen and in some cases insect resistance is now assayed.

If differences among crop varieties are substantial, breeding and selection for allelochemical resistance and weed resistance might become frequent practice. In some cases the use of interspecific or intergeneric hybrids might be of use. For example, rye is highly resistant to allelochemicals (not shown), while wheat is probably the most sensitive of grains tested (Section 3.1.2). Sets of rye addition lines, different wheat lines each containing one chromosome from rye, might be compared to see if particular rye chromosomes can contribute increased resistance to wheat. Rye may also be more toxic to weeds than is wheat; this could be examined using the same addition lines.

If substantial differences are identified, well-designed experiments could find the magnitude of the contribution of allelopathy to interference. No genetic approach to this difficult question has been reported.

Information on the relative toxicity and toxin resistance of different crop species and varieties might be very useful in setting up crop rotations. Such information could easily be found by examining effects of residues of all the common crops in a rotation on all the others under controlled field or laboratory conditions. The rotation could then be arranged to avoid following more toxic crops with more sensitive ones.

4.2 Introduction of foreign genes for detoxification

Many foreign genes have potential for introduction into plants to detoxify allelochemicals. Useful P450 genes other than *Cyp*1A1 and *Cyp*2A5 may be identified.

Preliminary research on the substrate ranges, among allelochemicals, of these enzymes would be useful. It is likely that substrates of plant P450 enzymes will be identified in the near future, and some of these may potentially be transferred between plant species either to increase toxicity of crop species or to increase their resistance to toxins produced by weeds.

Perhaps the best sources of foreign genes will be among fungi, which frequently grow on live or decaying plant material. Since phenolic phytoalexins are one of the plant's chief strategies for repelling fungal infection, successful pathogens may contribute excellent genes for the detoxification of this type of compounds.

This leads to potentially the largest drawback to this approach, namely, that the role of the phenylpropanoid pathway in disease resistance might somehow be compromised by the introduction of such genes. I believe that there is some risk where particular enzymes of narrow specificity are introduced to detoxify particular complex phenolic compounds which act both as allelochemicals and phytoalexins. Scopoletin in sunflower (*Helianthus annuus*) is an example. Sunflower makes scopoletin and ayapin in response to infection, but these are degraded in the presence of the successful sunflower pathogens *Phoma macdonaldii* or *Alternaria helianthi* (Tal and Robeson, 1986). Such species-specific pathogens might have genes useful for placing in plants subject to particular allelopathic stresses. For example, crop varieties following sunflower might be equipped with transgenes to detoxify scopoletin. If introduced into sunflower to reduce autotoxicity, however, such genes might increase its susceptibility to pathogens not normally successful on sunflower.

On the other hand, it might be possible in future to use such a strategy to change a plant's "recognition profile" to insects and pathogens to something less distinctive, thus confusing rather than attracting species-specific insects and pathogens. Where the main phenylpropanoid pathway is considered, this is probably a minimal hazard. Since some introduced genes would tend to hasten the movement of phenolic compounds through this pathway toward their end products, it might even enhance the speed with which a plant may repel infection. The question, however, is a serious one and deserves investigation.

In some cases another reason for caution exists. Crop and weed species are frequently closely related and sometimes identical. *Brassica campestris* is an example. A *B. campestris* plant with increased resistance to allelochemicals might become a more injurious weed due to the introduced resistance. For this reason it seems to me best that such a strategy should be limited to crops with little tendency to become weedy, or which are easily controlled by other means.

4.3 Resistance to artificial herbicides

Allelochemicals may be considered as low-potency, natural herbicides. Seen so, the question arises: why bother? Why not use a high-potency artificial herbicide in conjuction with a herbicide-resistant variety?

Farmers formerly controlled weeds almost entirely by cultivation practices. Effective herbicides were developed and gradually became popular. Their use has facilitated the strong current trends towards reduced till and no till farming. Reduced cultivation saves energy and time and reduces soil compaction and erosion. However, as presently practised it requires much herbicide. Herbicides carry costs in money, specialized equipment, and contamination of surface and ground water. Some appear to be of very low mammalian toxicity; others are not. In numerous cases biological activity of the breakdown products is not known. Herbicides may also be limited in application by the fact that the crop plant is susceptible. However, the large number of herbicides now available supplies something for most crop-weed combinations. Herbicide-resistant crops are another possible solution, especially where weed and crop are very closely related.

The development of herbicide resistance in crop plants has been a goal of great interest to biotechnologists and herbicide firms. Good levels of success have been reported in many crops (Mazur and Falco, 1989), although yield penalties are frequent. However, the crop itself may be a weed in other contexts, or the gene for herbicide resistance may be transferred to related weed species in some cases (Williamson, 1991). Also, weeds are capable of evolving their own herbicide resistance and in many cases have done so (Holt and LeBaron, 1990). Herbicide manufacturers intend that the extensive use of crops resistant to particular herbicides will cause an increase in the use of this herbicide rather than others or none. This, however, increases selective pressure on weed populations for high herbicide resistance, which ends the advantage of the resistant cultivar and potentially of the herbicide. Many weeds resistant to a single herbicide and several resistant to more than one are known. Among these are blackgrass (*Alopecurus myosuroides*) and annual ryegrass (*Lolium rigidum*), both of which inactivate several unrelated herbicides, possibly via an altered cytochrome P450 enzyme. In both cases the P450 inhibitor aminobenzotriazole partly restores herbicide activity (Kemp et al., 1990; Powles et al., 1990). A wild oat biotype recently identified in Alberta is resistant to two herbicides, triallate and difenzoquat, with no known common factor in their mode of action (J. O'Donovan, personal communication). Of 30 wild oat populations identified as triallate resistant, two were also diclofop resistant and one was also trifluralin resistant (Thai et al., 1985). The three classes of herbicides which affect acetolactate synthase (sulfonureas, imidazolinones, triazolopyrimidines) may be countered by increased expression of this one enzyme (Hartnett et al., 1991).

In general, it appears likely that more extensive use of herbicides will increase the incidence of resistance to them, which will limit their lifespans. Food and water supplies contaminated by herbicides are also increasingly unacceptable to consumers, while herbicide purchases are frequently a heavy item in farm accounts.

On the other hand, low-level increased tolerance of natural herbicides such as coumarin carries relatively little risk of appearing among weeds at increased levels. Without prohibiting the appearance of weeds, it would be expected to decrease their competitiveness and thence their interference with the crop. The balance of power, so to speak, between crop and weed is a delicate one. Slight shifts in this balance may cause large responses. For example, barley emergence five days before wild oats gives a 0% reduction in yield, while emergence five days later than wild oats gives a 23% reduction in yield, at wild oat density of 50 plants/m^2 (O'Donovan et al., 1985). Changes in allelopathic potential or tolerance could alter this balance favourably. Many of the factors which may act on crops interact. Roots reduced in length by allelopathic effects might interact with mild drought to reduce yield disproportionately, for example.

The development of allelochemical tolerant crops, if successful, should provide several advantages. Weeds should be reduced in size and number, and important weeds might be reduced to unimportant ones. The crops might be less susceptible to certain other stresses, such as drought. Carryover of phenolic compounds from one season to the next should not increase. Financially, after the initial switch to allelochemical tolerant varieties, no further costs would be incurred and some others such as herbicide and cultivation costs should decrease.

It is not possible to get rid of every weed. It is expensive to try. Often it may not be useful. For many purposes weeds do not damage crop quality. Wild oats is superior to domestic oats in protein content (Rines et al., 1980) and is good animal feed- indeed, is presently marketed as a breakfast cereal. In some cases low numbers of weeds increase crop yield (Altieri, 1988; Rice, 1984). In many countries weeds are eaten before the crop ripens, or if it fails. Weeds assist in erosion control (Altieri, 1988) and appear to reduce populations of both specialized and non-specialized phytophagous insects and to increase those of their predators (Androw, 1988). Where closely related to crops, weeds may serve as a gene bank. The "gold standard" of fields perfectly free of weeds is artificial and for some purposes might profitably be re-examined.

4.4. Conclusion

This thesis explores several possible ways to influence allelopathic interactions through genetic manipulations, with the long-term goal of creating crop varieties which are less injured by the presence of weeds. Such crop varieties should yield well in the absence of intensive herbicide use or cultivation. Since this question has not been addressed previously, it is necessarily broad in scope, representing beginnings rather than complete treatments of the questions explored.

Two approaches to increase the resistance of crop species to allelopathic effects of weeds were investigated. The first, the identification of variability within species in response to common allelopathic chemicals and to weed extracts, suggests the feasibility of selection for resistance to allelochemicals and hence to interference by weeds. The second, the introduction of foreign genes expected to detoxify some common allelochemicals, was successful to the point of demonstrating transcriptional activity of introduced genes. However, the expected enzyme activities and phenotypic effects did not occur. Various possible reasons for this unexpected result exist. These or other genes may prove useful in future for developing crop varieties which are hardier to weeds.

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Appendix 1.

Plant DNA miniprep (modified from Dellaporta *et al*, 1985).

1. For leaf DNA, punch 3 8 mm discs from young leaves with cork borer. Place leaves in Eppendorf tube on ice. For callus or suspension cells, sop 300-400 mg cells on filter paper and place in Eppendorf tube on ice. Add 0.5 ml buffer (100 mM Tris HCl pH 8.0, 50 mM EDTA, 0.5 M NaCl, 1 µg/ml β-mercaptoethanol, 5% by volume of 20% SDS).
2. Grind thoroughly with fitted pestle. May be hooked up to power drill. Add 0.5 ml more of same buffer.
3. Heat 65°C for 10 minutes. Add 0.33 ml of 5 M potassium acetate, pH 5.2.
4. Hold on ice for 20 minutes. Spin at top speed in Eppendorf minicentrifuge for 6 minutes.
5. Carefully transfer supernatant to new tube. May need to respin. Take not more than 0.9 ml. To 0.9 ml DNA solution add 0.6 ml isopropanol. Hold at -20°C for 30 minutes.
6. Spin 12 minutes. Wash DNA pellet with 70% ethanol.
7. Drain or vacuum fairly dry. Resuspend in about 100 µl water. Add restriction enzymes and 3 µl of 10 mg/ml RNase A. Digest overnight.
8. Reduce volume by ethanol/sodium acetate precipitation if necessary. Run on gel.